

Identification and characterisation of novel Wnt regulators in
colorectal cancer

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Declaration

I Pedro Rafael da Costa Antas confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Colorectal cancer (CRC) is a multistep mutagenic process that provides tumour cells with a growth advantage for clonal expansion. The Wnt signalling pathway is crucial for regulating ISC maintenance and differentiation, whilst aberrant Wnt activation is a hallmark of human CRC. Recent advances in genome-scale analysis of large cohorts of CRCs have uncovered a large number of novel mutations that had not been previously characterised. Interestingly, hierarchical clustering analyses of the related expression data reveals a cluster of differentially expressed genes that is enriched in the previously reported ISC markers, Wnt targets and/or Wnt inhibitors. We hypothesised that some of the genes within the cluster are expressed at the intestinal crypt bottoms to antagonise Wnt signalling to form part of a negative feedback mechanism for ISC homeostasis. The aim of my project was to screen for novel Wnt regulators within this gene cluster.

Comprehensive analysis of the cluster identified two potential Wnt regulators: the SH3 domain-binding protein 4 (SH3BP4) and the serine/threonine kinase ZAK/MAP3K20. Here we show that both *Sh3bp4* and *Zak* are expressed at the ISC compartment and are enriched in *Apc*-mutated tumours. Deletion of *Zak* enhances radiation-induced intestinal regeneration, suggesting a growth inhibitory role of ZAK in the intestine. Loss of SH3BP4 *in vivo* exacerbates the *Apc^{min}* tumour phenotype by promoting the total number of adenomas and high-grade dysplasia. Mechanistically, ZAK and SH3BP4 suppresses Wnt signalling downstream of β -catenin phosphorylation. The data revealed novel roles for both ZAK and SH3BP4 in intestinal homeostasis and tumourigenesis via Wnt/ β -catenin signalling regulation.

Taken together, the findings highlight the crucial role that these newly identified genes play in Wnt negative feedback mechanisms in intestinal regeneration and tumourigenesis, which may provide new insights into targeted therapeutic intervention.

Impact Statement

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. In-depth characterisation of the molecular mechanisms underlying CRC development is important for therapeutic advances. Comprehensive genetic studies over the past few decades have demonstrated the crucial role of Wnt signalling in a variety of cancers and other diseases. In particular, aberrant Wnt activation is the primary cause of most CRCs. However, there is still lack of efficient drugs to target Wnt signalling in the clinic. Recent advances in high-throughput sequencing have further revealed many novel recurrent Wnt pathway mutations in addition to the well-characterized *APC* mutation in CRC. Understanding how these mutations contribute to cancer development may open exciting avenues for drug targeting and personalised medicine.

Based on the combined knowledge of Wnt signal regulation during both normal ISC homeostasis and CRC progression, we have identified two novel Wnt inhibitors - SH3BP4 and ZAK. Our results suggest that both SH3BP4 and ZAK are direct Wnt transcriptional targets, which function to inhibit Wnt signalling as a negative feedback mechanism for normal ISC homeostasis. Functionally, loss of *Sh3bp4* promotes intestinal tumour initiation and progression, whereas *Zak* depletion promotes intestinal regeneration. These findings contribute to a better understanding of the Wnt signal regulation during ISC homeostasis, regeneration and tumorigenesis in the field of stem cell and cancer biology.

In this project, we have generated novel ZAK knock-out mouse models using state-of-the-art CRISPR/Cas9 targeting in different mouse strain backgrounds. The development of the new mouse models provides a significant contribution to the broader scientific community, allowing the study of the role of ZAK in different contexts. In fact, our new *Zak*-null mice are currently being used by another laboratory to gain insight into the functional significance of ZAK in muscle hypertrophy. In addition, we have also generated *Sh3bp4-flag* knock-in mice, which allow us to locate the tagged protein expression in primary tissues to overcome the lack of reliable, commercially available antibodies. The endogenous tagged protein may further be used for biochemical studies to identify other molecular interplays, such as immunoprecipitation.

Finally, scientific communication is a *sine qua non* for progress in science. During my PhD, I co-authored a review titled “*Targeting Wnt signaling in colorectal cancer*” in 2015, I presented a poster at the International Wnt signalling Gordon Research Conference in 2017, and I am also an author on a paper published in Cell Reports. Furthermore, we intend to submit two research articles to communicate our findings with the broader scientific community.

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Doing a PhD is a hard job right from the start. First you have to decide on the topic, then the leading institution to apply to and finally – the hardest part of all – who to approach to be your supervisor. I would like to thank, above all, my supervisor Dr. Vivian Li. I am grateful to her for having me in her laboratory, for the invaluable scientific discussions, for believing in my skills and encouraging me to go ahead with my ideas. I strongly believe that you do not need a supervisor to meet with you every day but rather one that, no matter what, puts you at the top of their agenda every time you face a problem. I was fortunate enough to have exactly that. Next, I am grateful to all my fellow labmates. Together, we started the lab. We unpacked the pipettes and all the equipment. We optimised protocols and discussed ideas. Together, we made mistakes but we learned from them and thrived. No one could have done a better job.

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Table of Contents

Abstract	3
Impact Statement	4
Acknowledgement	6
Table of Contents	8
Table of figures	10
List of tables	12
Abbreviations	13
Chapter 1. Introduction	16
1.1 Intestinal architecture and stem cell biology	17
1.2 Wnt signalling pathway	21
1.2.1 β -catenin independent pathways	21
1.2.2 Wnt/ β -catenin pathway	22
1.2.3 Receptors and ligands for Wnt/ β -catenin signalling	24
1.2.4 The β -catenin destruction complex	28
1.2.5 Wnt signalling in the nucleus	29
1.2.6 Wnt/ β -catenin inhibitors	30
1.2.7 Wnt/ β -catenin negative feedback loop	32
1.3 Notch signalling	34
1.4 EGFR signalling	36
1.5 BMP signalling	38
1.6 Colorectal cancer biology	39
1.6.1 APC mutations in colorectal cancer	40
1.6.2 CRC molecular subtypes	41
1.6.3 Cells of origin of CRC	44
1.6.4 Mutations in Wnt signalling components in CRC	45
1.6.5 Crosstalk between Wnt/ β -catenin signalling and other pathways in CRC	48
1.7 Hypothesis	49
1.8 Aim	52
Chapter 2. Materials & Methods	53
2.1 Cell culture, transfection and TOPFlash assay	53
2.2 Cells gene Editing by CRISPR/Cas9 System	53
2.3 DNA cloning and plasmids	55
2.4 Immunoblotting and immunoprecipitation	56
2.5 Crypts/Villi fractionation	57
2.6 Real-time quantitative RT-PCR	57
2.7 Immunofluorescence	58
2.8 Immunohistochemistry	58
2.9 RNA <i>in situ</i> hybridization	58
2.10 Intestinal organoid culture and imaging	59
2.11 Mouse experiments	59
2.11.1 Mouse generation by CRISPR/CAS9-mediated genome engineering	61
2.11.2 Genotyping and PCR amplification	61

2.11.3 EdU injections	62
2.11.4 Induction of <i>Cre</i>	62
2.11.5 Radiation injury model	62
2.12 Statistical analysis	62
Chapter 3. Screening for novel Wnt regulators in colorectal cancer	64
3.1 Introduction	64
3.2 Results	66
3.2.1 TCGA hierarchical clustering reveals a “Stem-cell/Wnt cluster”	66
3.2.2 Identification of novel putative Wnt regulators by comparing the Stem Cell/Wnt cluster with other microarray data	69
3.2.3 Expression pattern of the selected candidate genes <i>Sh3bp4</i> and <i>Zak</i> in the intestine	73
3.2.4 Validation of <i>Zak</i> and <i>Sh3bp4</i> as Wnt target genes	77
3.2.5 Mutations identified in public databases	79
3.3 Discussion	81
Chapter 4. The ZAK kinase is a Wnt negative regulator expressed by stem cells 83	
4.1 Introduction	83
4.2 Results	86
4.2.1 ZAK is a negative regulator of Wnt/ β -catenin signalling	86
4.2.2 The Wnt negative regulator ZAK functions downstream of β -catenin phosphorylation/ubiquitination	90
4.2.3 <i>Zak</i> -null mouse characterisation	94
4.2.4 Loss of <i>Zak</i> promotes intestinal regeneration after radiation-induced injury 100	
4.3 Discussion	107
Chapter 5. SH3BP4 inhibits intestinal tumour initiation and growth by negative regulation of Wnt signalling.....	111
5.1 Introduction	111
5.2 Results	113
5.2.1 SH3BP4 is a negative regulator of Wnt/ β -catenin signalling	113
5.2.2 SH3BP4 inhibits Wnt signalling downstream of β -catenin phosphorylation/ubiquitination.....	117
5.2.3 SH3BP4 interacts with APC and β -catenin.....	123
5.2.4 Loss of SH3BP4 promotes intestinal stem cell proliferation	126
5.2.5 Loss of SH3BP4 exacerbates <i>Apc</i> -mediated intestinal tumour initiation and progression	135
5.3 Discussion	140
Chapter 6. General discussion.....	142
6.1 Wnt/ β -catenin negative feedback mechanisms in the ISC compartment.....	142
6.2 Mechanistic role of ZAK and SH3BP4 and their therapeutic implications	145
6.3 Future directions	149
Chapter 7. Appendix 1.....	153
Reference List.....	154

Table of figures

Figure 1.1 Intestinal biology in homeostasis and disease.	18
Figure 1.2 Schematic diagram showing overview of Wnt/ β -catenin signalling pathway.	24
Figure 1.3 Intestinal homeostasis and Notch signalling.	35
Figure 1.4 EGFR signalling in intestine.	37
Figure 1.5 Molecular subtypes of colorectal cancer.	43
Figure 1.6 Schematic representation of the project hypothesis.	51
Figure 3.1 TCGA hierarchical clustering reveals “Stem-cell/Wnt cluster”	68
Figure 3.2 Expression pattern of the potential Wnt regulator candidates in stem cell/Wnt-related microarray studies.	71
Figure 3.3 Schematic diagram of shortlisting strategy for putative Wnt regulators.	73
Figure 3.4 Expression pattern of the selected candidate genes, <i>Sh3bp4</i> and <i>Zak</i> , in the intestine.	75
Figure 3.5 ZAK and SH3BP4 protein expression analysis.	76
Figure 3.6 <i>Zak</i> and <i>Sh3bp4</i> expression are increased in <i>Apc^{min}</i> adenomas.	78
Figure 3.7 Mutations of <i>ZAK</i> and <i>SH3BP4</i> found in human colorectal cancer.	80
Figure 4.1 ZAK inhibits Wnt/ β -catenin signalling.	89
Figure 4.2 ZAK inhibits Wnt signalling downstream of β -catenin phosphorylation and ubiquitination.	92
Figure 4.3 The Wnt inhibitory role of ZAK is independent of MAPK signalling.	93
Figure 4.4 Generation of <i>Zak</i> ^{-/-} mice by CRISPR/Cas9 genome editing.	97
Figure 4.5 <i>Zak</i> ^{-/-} intestine show normal stem cell proliferation and differentiation.	98
Figure 4.6 Depletion of <i>Zak</i> promotes intestinal regeneration after radiation-induced injury.	103
Figure 4.7 Depletion of <i>Zak</i> increases expression of ISC markers and Wnt targets after radiation-induced injury.	104
Figure 4.8 <i>Zak</i> ^{-/-} mice show normal intestinal phenotype 6 days after radiation-induced injury.	105
Figure 4.9 Intestinal crypts isolated from <i>Zak</i> ^{-/-} mice demonstrate higher organoid formation efficiency as compared to WT.	106
Figure 5.1 Deletion of SH3BP4 activates Wnt/ β -catenin signalling.	115

Figure 5.2 SH3BP4, but not MACC1, inhibits Wnt/ β -catenin signalling.	116
Figure 5.3 SH3BP4 inhibits Wnt signalling downstream of β -catenin phosphorylation.	118
Figure 5.4 SH3BP4-mediated inhibition of Wnt/ β -catenin signalling is dependent on the ZU5 domain.	120
Figure 5.5 Activation of Wnt/ β -catenin signalling in Δ SH3BP4 is independent of mTOR pathway activation.	122
Figure 5.6 SH3BP4 is predominantly localised at perinuclear membrane and nucleus.	124
Figure 5.7 SH3BP4 interacts with APC and β -catenin.	125
Figure 5.8 Characterisation of <i>Sh3bp4</i> ^{fl/fl} <i>Villin</i> ^{CreERT2} mice 25 days after SH3BP4 loss.	127
Figure 5.9 Loss of <i>Sh3bp4</i> (25 days) increases expression of ISC markers.	130
Figure 5.10 <i>Sh3bp4</i> deletion (25 days) increases lysozyme expression and crypt proliferation.	131
Figure 5.11 Characterisation of <i>Sh3bp4</i> ^{fl/fl} <i>Villin</i> ^{CreERT2} mice 3 months after SH3BP4 loss.	134
Figure 5.12 Loss of SH3BP4 exacerbates the <i>Apc</i> ^{min} phenotype.	137
Figure 5.13 <i>Sh3bp4</i> ^{fl/fl} <i>Villin</i> ^{CreERT2} <i>APC</i> ^{min} adenomas display same apoptosis index compared to control <i>APC</i> ^{min} adenomas.	138
Figure 5.14 Characterisation of goblet cell and Paneth cell differentiation in <i>Sh3bp4</i> ^{fl/fl} <i>Villin</i> ^{CreERT2} <i>APC</i> ^{min} mice.	139
Figure 6.1 Wnt inhibitors and CRC.	143
Figure 6.2 ZAK inhibits Wnt signalling independently of MAPK pathway activation.	147
Figure 6.3 SH3BP4 inhibitory role in Wnt signalling.	149
Figure 6.4 Graphical summary of potential mechanisms of action.	152

List of tables

Table 1. List of primers used.	54
Table 2. List of antibodies	55
Table 3. List of transgenic mice used	60
Table 4. List of 26 genes with 1.5-fold upregulated expression after <i>Apc</i> deletion	72

Abbreviations

APC	adenomatous polyposis coli
BMP	bone morphogenic protein
Bp	base pair
Brg-1	Brahma-related gene-1
CBC	crypt base columnar
CBP	CREB-binding protein
CID	β -catenin inhibitory domain
CK1	casein kinase 1
CMS	consensus molecular subtypes
CRC	colorectal cancer
CRISPR	clustered regularly interspaced short palindromic repeats
CtBP	carboxyl-terminal binding protein
DAAM1	DVL associated activator of morphogenesis 1
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DKK	dickkopf
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DVL	dishevelled
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EIF3E	eukaryotic translation initiation factor 3
ERK	extracellular signal-regulated kinase
ESCs	embryonic stem cells
Evi	evenness interrupted
FAP	familial adenomatous polyposis
FBS	foetal bovine serum
FGFR	fibroblast growth factor receptor

FZD	frizzled receptors
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3	glycogen synthase kinase 3
GTPase	hydrolyse guanosine triphosphate
H&E	eosin-haematoxylin staining
Hes	hairy and Enhancer of Split
HET	heterozygous
HGD	high grade dysplasia
HOM	homozygous
IHC	immunohistochemistry
IP	immunoprecipitation
ISC	intestinal stem cell
ISH	<i>in situ</i> hybridization
JNK	c-Jun N-terminal kinase
Kb	kilobase
KI	knock-in
Krm	Kremen
LEF	lymphoid enhancer-binding factor
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5
LRP	low-density lipoprotein receptor-related protein
LZ	leucine zipper domain
MACC1	metastasis-associated in colon cancer protein 1
MAPK	mitogen-activated protein kinase
MED12	mediator subunit 12
MLK	mixed-lineage kinases
MSI	microsatellite instability
MSS	microsatellite stable
mTOR	mammalian target of rapamycin
NEHJ	non-homologous end joining
NES	nuclear export sequences
NICD	notch intracellular domain
NTR	netrin-related motif
PAS	periodic acid–Schiff staining
PCP	planar cell polarity

PCR	polymerase chain reaction
PFA	paraformaldehyde
PKC	protein kinase C
PLC	G-protein phospholipase C
PTPRK	receptor type tyrosine protein phosphatase
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
RasGEFs	ras guanine nucleotide exchange factors
RNA	ribonucleic acid
RNF43	E3 ubiquitin ligases RING finger 43
ROCK	rho-associated coiled-coil-containing protein kinase
ROR	tyrosine-protein kinase transmembrane receptor
RSPO	R-spondin
SAM	sterile-aa-motif
SCNA	somatic copy number alterations
SDS	sodium dodecyl sulfate
SFRPs	secreted frizzled-related proteins
sgRNA	single guide RNA
SH3BP4	SRC homology 3 domain-binding protein 4
SOST	sclerostin
TA	transit-amplifying cells
TBP	TATA-binding protein
TCF	T cell transcription factor
TCGA	Cancer Genome Atlas
TfR	transferrin receptor
TGF	β -transforming growth factor-beta
WIF-1	Wnt-inhibitory factor 1
Wntless	Wis
WT	wild-type
WTX	adenomatous polyposis coli membrane recruitment 1
ZAK	mixed-lineage zipper sterile- α -motif kinase
ZNRF3	zinc and ring finger 3

Chapter 1. Introduction

The gastrointestinal tract plays a crucial role in nutrient absorption, and provides a physical barrier to pathogens and toxic compounds present in the intestinal lumen. The constant exposure to the hostile environment results in continual cell loss that needs to be continually replaced. Replenishment of the intestinal epithelium is supported by intestinal stem cells (ISCs) that are located at the bottom of intestinal crypts. The high proliferation rate in this region makes the intestine a very attractive model for the study of stem cell proliferation and differentiation. Intestinal epithelial homeostasis is tightly regulated by multiple signalling pathways, including Wnt, Notch, epidermal growth factor (EGF) and bone morphogenetic protein (BMP) signals. Wnt signalling constitutes the key pathway to maintain the stem cell population and drive proliferation (Korinek et al., 1998). EGF signals are essential for the high mitotic cell cycles of the transit-amplifying (TA) cells upon engagement of their receptors (EGFRs) (Wong et al., 2012). Notch signalling is believed to determine the absorptive or secretory lineage differentiation of early progenitor cells (Fre et al., 2005). The BMP pathway is active in the villus compartment to drive differentiation and repress “stemness” genes (Haramis et al., 2004). The role of these pathways in intestinal homeostasis is discussed in more detail later in this chapter. Many other signalling pathways, such as Eph/Ephrin, Hedgehog and Hippo signalling, are also important in regulating intestinal homeostasis, and their roles have been extensively addressed elsewhere and will not be discussed here (for reviews see (Beumer and Clevers, 2016; Meran et al., 2017; Sailaja et al., 2016)).

The Wnt signalling pathway is the gatekeeper for normal intestinal homeostasis, whilst aberrant activation is the major cause of human colorectal cancer (CRC). Activating mutations in the Wnt pathway initiate the formation of intestinal adenomas (Fearon, 2011), which require subsequent accumulation of other driver mutations for malignant transformation (Fearon and Vogelstein, 1990). This chapter summarises the current understanding of intestinal stem cell biology and the signalling pathways involved, with the major focus placed on the Wnt signalling pathway. The recent advances in CRC biology and different Wnt activating mechanisms are also addressed in detail.

1.1 Intestinal architecture and stem cell biology

The intestine is anatomically divided into the small intestine and the colon. The small intestine can be further subdivided into the duodenum, jejunum and ileum regions and is organised into crypts of Lieberkühn and villi (Figure 1.1A). The colonic epithelium has two distinctive features when compared with the small intestine: 1) colon has a flat epithelial surface without protruding villi, and 2) Paneth cells found at the base of small intestinal crypts are absent from colon (Figure 1.1B). However, recent studies subsequently revealed that cKIT⁺ cells and *Reg4*⁺ deep crypt secretory cells located at the crypt bases function as Paneth-like niche cells to support stem cells in colon (Rothenberg et al., 2012; Sasaki et al., 2016).

The intestinal epithelium is the most regenerative tissue in adult mammals (Heath, 1996). Given that the entire crypt-villus architecture is renewed approximately every 5-7 days, it represents one of the best models for the study of epithelial cell development. The high cell turnover is supported by ISC located at the crypt bottoms. ISCs divide and produce rapidly proliferating daughter transit-amplifying (TA) cells. The TA progenitor cells continue to divide and migrate upwards towards the villi, where they differentiate. It is believed that the early progenitor cells at the TA compartment retain a certain degree of plasticity during regeneration (Tetteh et al., 2016), whilst cells reaching the crypt-villus junction will terminally differentiate into one of the two main epithelial cell lineages: absorptive or secretory. The absorptive lineage comprises enterocytes, whilst the secretory lineage includes mucous-secreting goblet cells, hormone-secreting enteroendocrine cells and Paneth cells (Noah et al., 2011). Paneth cells provide innate immunity and secrete anti-microbial defensin peptides (such as cryptdins) and digestive lysozymes. These cells have a relatively low turnover rate and they are the only differentiated cell type that migrates back towards the crypt bottom due to repulsive EphB3/EphrinB interactions (Batlle et al., 2002). There are other differentiated cell types present in the epithelium including Tuft cells and M cells, although their lineage and function are not yet fully characterised yet (Gerbe et al., 2012; Miller et al., 2007).

When the differentiated cells reach the tip of the villus, they undergo apoptosis and are shed into the intestinal lumen (Hall et al., 1994).

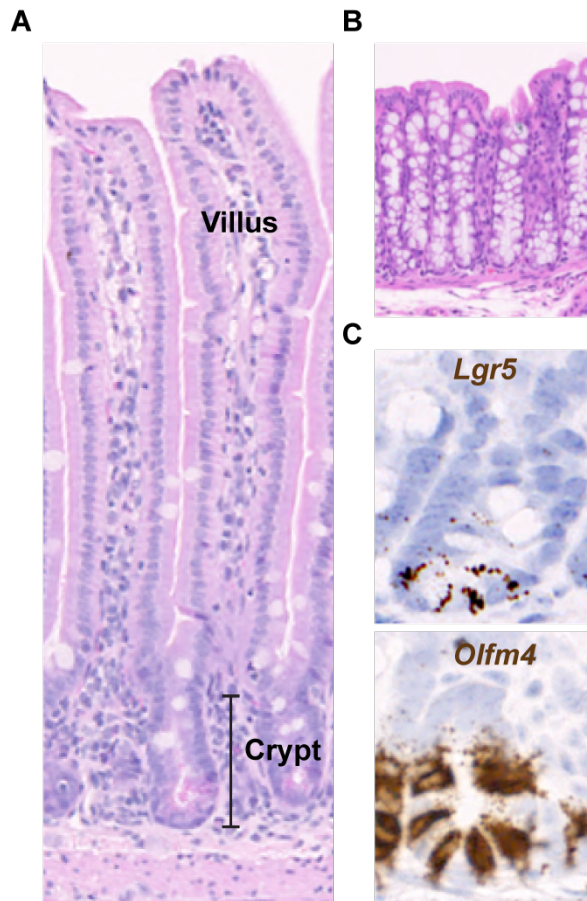


Figure 1.1 Intestinal biology in homeostasis and disease.

(A) Eosin-haematoxylin staining (H&E) of a mouse small intestine showing villus and crypt structure. (B) H&E of a mouse colonic epithelium. (C) Expression pattern of the two stem cell specific markers *Lgr5* (upper panel) and *Olfr4* (lower panel) using RNAscope in situ hybridization

Intestinal stem cells. The existence of the “stem cell zone” composed of columnar

cycling cells, intercalated between Paneth cells, was first suggested more than 40 years ago by Cheng and Leblond (Cheng and Leblond, 1974a, b). The development of clonal marking techniques provided further evidence that the crypt base columnar (CBC) cells were the ISCs that divide and give rise to all intestinal epithelial cell types (Bjerknes M, 1999). This was later proven to be correct using *in vivo* lineage tracing experiments after the discovery of the first CBC cell marker, leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) (Barker et al., 2007). The development of multicolour lineage tracing techniques further demonstrated that ISC

typically divide symmetrically to generate two daughter cells (Lopez-Garcia et al., 2010; Schepers et al., 2011; Snippert et al., 2010b). The fate of these ISC daughter cells is believed to be stochastic depending on complex interplay between internal and external signals from the surrounding niche, which either regenerates another ISC via self-renewal, or a more differentiated progenitor. Previous studies have shown that subepithelial myofibroblasts contribute to crypt niche signalling by secreting various growth factors and cytokines that promote epithelial proliferation (Mills and Gordon, 2001; Powell et al., 1999). Cell-cell interaction with Paneth cells has also been suggested as a key determining factor for “stemness”, since they secrete various essential stem cell niche factors such as EGF, Wnt3 and Notch ligand (Sato et al., 2011). However, Paneth cells are unlikely to be the only source of niche signals as intestinal homeostasis can be maintained in their absence (Durand et al., 2012; Kima et al., 2012), probably due to other redundant sources of Wnt proteins (Farin et al., 2012).

As mentioned above, *Lgr5* is exclusively expressed by CBC cells (Figure 1.1C). Lineage tracing experiments using the *Lgr5-EGFP-IRES-CreERT2; R26R-LacZ* reporter mouse model confirmed that the *Lgr5*⁺ cells are the main pool of self-renewing ISCs that can give rise to all differentiated epithelial cell types (Barker et al., 2007). The *Lgr5*⁺ ISCs are located at position 1-4 (counting up from the crypt base) and are evenly distributed between Paneth cells. The ability of sorted-*Lgr5*⁺ cells to generate three-dimensional intestinal organoid cultures *in vitro* with full self-renewal and differentiation potential provided further supportive evidence of their stemness (Sato et al., 2009). Several other markers such as *Ascl2* and *Olfm4* have also been reported to be exclusively expressed by CBC cells (van der Flier et al., 2009a; van der Flier et al., 2009b) (see *Olfm4* expression in (Figure 1.1C). In addition to this fast-proliferating stem cells, a label-retention study performed by Potten in 1974 showed evidence of a second pool of quiescent/slow-cycling stem cells (Potten, 1977). More recent lineage tracing experiments with histone 2B yellow fluorescent protein (H2B-YFP) (Buczacki et al., 2013) and green fluorescent protein (H2B-GFP) (Roth et al., 2012) have confirmed the existence of these long-lived cells population that can act as stem cells in regenerative processes.

Several studies have suggested the presence of other stem cell markers such as *Bmi1* (Sangiorgi and Capecchi, 2008), *Hopx* (Takeda et al., 2011), *mTert* (Montgomery et al., 2011), and *Lrig1* (Powell et al., 2012). However, comprehensive

expression analysis of these markers demonstrated that most of them are expressed broadly at the base of the crypt as well as in *Lgr5*⁺ cells (Itzkovitz et al., 2011; Munoz et al., 2012). As a matter of fact, recent evidence supported the idea that cells at the crypt of intestine have great plasticity and could change their expression profile and contribute to the regenerative process following tissue insults. For example, ablation of the *Lgr5*⁺ ISC population is tolerated during intestinal homeostasis by enhanced rates of proliferation of *Bmi1*⁺ cells (Hua Tian et al., 2011; Yan et al., 2012). This same observation has been made for *Hopx*-expressing cells (Takeda et al., 2011). A more recent study further showed that *Bmi*⁺ cells are enteroendocrine progenitors and goblet cell precursors, which can rapidly dedifferentiate in response to ablation of *Lgr5*⁺ ISCs (Jadhav et al., 2017). Several other studies have shown that secretory progenitors (van Es et al., 2012b) and enterocytes (Tetteh et al., 2016) can also support regeneration of intestinal epithelium following damage to *Lgr5*⁺ ISC population. However, despite the fact that *Lgr5*⁺ seem to be dispensable for homeostasis of the intestine, they are fundamental for intestinal regeneration upon irradiation-induced injury (Metcalf et al., 2014), showing that in certain insults, the cells in the stem cell niche are not able to compensate *Lgr5*⁺ cells loss.

Taken together, the recent evidence supports the notion of a high degree of plasticity in the intestinal crypt where earlier progenitors can dedifferentiate to produce stem cell-like properties in response to tissue insults.

Wnt signalling is the key pathway to drive ISC maintenance, proliferation and differentiation of the epithelial cells (Wetters et al., 2002). Characterisation of the expression patterns of various Wnt components in the intestine demonstrated that the Wnt pathway is non-uniformly active along the crypt-villus axis with the highest activity at the crypt bottoms (Gregorieff et al., 2005). Importantly, transcriptomic analysis of Wnt/T cell factor (TCF) targets identified *LGR5* as a Wnt target gene (Van der Flier et al., 2007), which subsequently led to the discovery of *LGR5* as a stem cell marker (Barker et al., 2007). In the next section, I will discuss the Wnt signalling pathway in detail and summarise the main studies regarding the role of Wnt signalling in the intestine.

1.2 Wnt signalling pathway

The Wnt signalling pathway is highly conserved throughout the animal kingdom and plays principal regulatory roles in many developmental and biological processes (Clevers, 2006; Logan and Nusse, 2004). Wnt signalling is also found to be aberrantly activated in many human diseases, including cancers and metabolic disorders (Clevers and Nusse, 2012). In the adult intestine, it is well established that Wnt signalling plays complementary roles in physiology and pathology: in health, it maintains crypt stem cell compartments, but, when activated by mutation/s, it is the cause of CRC. Wnt signalling is commonly divided into β -catenin dependent (also known as canonical) and β -catenin independent (non-canonical or alternative) pathways. Here, I provide an overview of both pathways and discuss the canonical Wnt pathway in detail at different subcellular levels of the signalling cascade: Wnt ligands and receptors, cytoplasmic destruction complex and nucleus.

1.2.1 β -catenin independent pathways

The mechanisms by which Wnt proteins regulate different pathways have yet to be fully characterised. However, cumulative evidence suggests that initiation and diversification of the Wnt signalling responses is determined at the level of ligands, co-receptors and cytoplasmic effectors. Interestingly, β -catenin independent pathways seem to be activated by specific Wnt ligands, such as Wnt5a (Gao et al., 2011; Moon et al., 1993) and Wnt11 (Heisenberg et al., 2000). On the other hand, several other studies suggest the existence of cross-reactivity of the Wnt-frizzled receptors (FZD) interactions, whilst only limited evidence supports the formation of specific ligand-receptor pairs. It is possible that the specificity of ligand-receptor interactions is most likely conferred by cellular context as determined by the expressed receptors and signal transducers rather than different properties of the Wnt ligands themselves. There are two distinct β -catenin independent intracellular cascades: Wnt/planar cell polarity (PCP) signalling and Wnt/Calcium signalling.

Wnt/PCP signalling controls various aspects of cell migration (Castanon et al., 2013) and polarity (Wu et al., 2013) that affect tissue morphogenesis. During

Wnt/PCP signalling, Wnt ligands bind to the tyrosine-protein kinase transmembrane receptor1/2 (ROR1/2)-FZD receptor complex (Kelley, 2008; Krausova and Korinek, 2013) to recruit and activate Dishevelled (DVL) (Tree et al., 2002). DVL binds to the hydrolyse guanosine triphosphate (GTPase) ras homolog gene family member A (RhoA), a key regulator of cytoskeletal architecture (Habas et al., 2001), through the DVL associated activator of morphogenesis 1 (DAAM1) protein. The Wnt-induced DVL-Rho complex triggers activation of the rho-associated coiled-coil-containing protein kinase (ROCK) and c-Jun N-terminal kinase (JNK). Moreover, DVL can also activate small GTPases ras-related C3 botulinum toxin substrate 1 (Rac1) for JNK signalling activation. This leads to transcriptional responses via activating transcription factor 2 (ATF) (Zallen, 2007).

Wnt/Ca²⁺ signalling, on the other hand, is triggered by G-protein phospholipase C (PLC). The activation of PLC upon engagement of the ROR1/2-FZD receptor complex leads to intracellular calcium fluxes that activate calcium/calmodulin-dependent kinase II (CamKII), protein kinase C (PKC) and calcineurin (Sheldahl et al., 2003), which subsequently activates downstream Ca²⁺-dependent transcriptional responses.

The Wnt/PCP and Wnt/Ca²⁺ signalling branches have been implicated in inflammation and promotion of cancer (Dissanayake et al., 2007; Wang, 2009). Although intestinal midgut elongation has been reported upon deregulation of alternative Wnt signalling pathways (Cervantes et al., 2009; Yamada et al., 2010), little is known about the role of non-canonical Wnt signalling in intestinal homeostasis and disease. The β -catenin independent Wnt signalling has been extensively reviewed previously and will not be addressed in detail here (Kikuchi et al., 2011; van Amerongen, 2012).

1.2.2 Wnt/ β -catenin pathway

β -catenin is the key downstream effector of canonical Wnt/ β -catenin signalling. The β -catenin level is tightly regulated by the cytoplasmic β -catenin destruction complex that consists of the scaffold proteins AXIN, adenomatous polyposis coli (APC), casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3)

(Figure 1.2, left). In the absence of Wnt ligands, the destruction complex phosphorylates β -catenin and targets it for proteosomal degradation upon ubiquitination mediated by β -TrCP, a component of the E3 ubiquitin ligase complex (Aberle et al., 1997; Liu et al., 2002). In the nucleus, the transcriptional repressor Groucho binds to the TCF transcription factor/lymphoid enhancer-binding factor (LEF) transcription complex to inhibit Wnt target gene transcription (Cavallo et al., 1998; Roose et al., 1998). When the Wnt ligands engage with FZD and the low-density lipoprotein related protein 5/6 (LRP5/6) co-receptor complex, the destruction complex is recruited to the membrane, where β -TrCP is dissociated from the complex (Li et al., 2012). As a consequence, β -catenin is no longer ubiquitinated and degraded, leading to saturation of the destruction complex. Alternative models of destruction complex inhibition have been suggested, such as the dynamic regulation of β -catenin phosphorylation, and AXIN-complex assembly mediated by phosphorylation (Hao et al., 2012; Hernández et al., 2012). Consequently, the newly synthesized β -catenin accumulates in the cytoplasm and is translocated into the nucleus. Nuclear β -catenin displaces the Groucho repressor to form the active β -catenin/TCF/LEF transcriptional complex, resulting in Wnt target gene transcription (Molenaar et al., 1996) (Figure 1.2, right).

Constitutive Wnt/ β -catenin signalling activation is a frequent event in many cancer types. In particular, mutations in the negative Wnt/ β -catenin signalling regulators are the hallmark of the majority of human CRCs (Nagase and Nakamura, 1993). Therefore, for the purposes of my thesis, I will only focus on exploring the Wnt/ β -catenin signalling pathway in detail in the following sections.

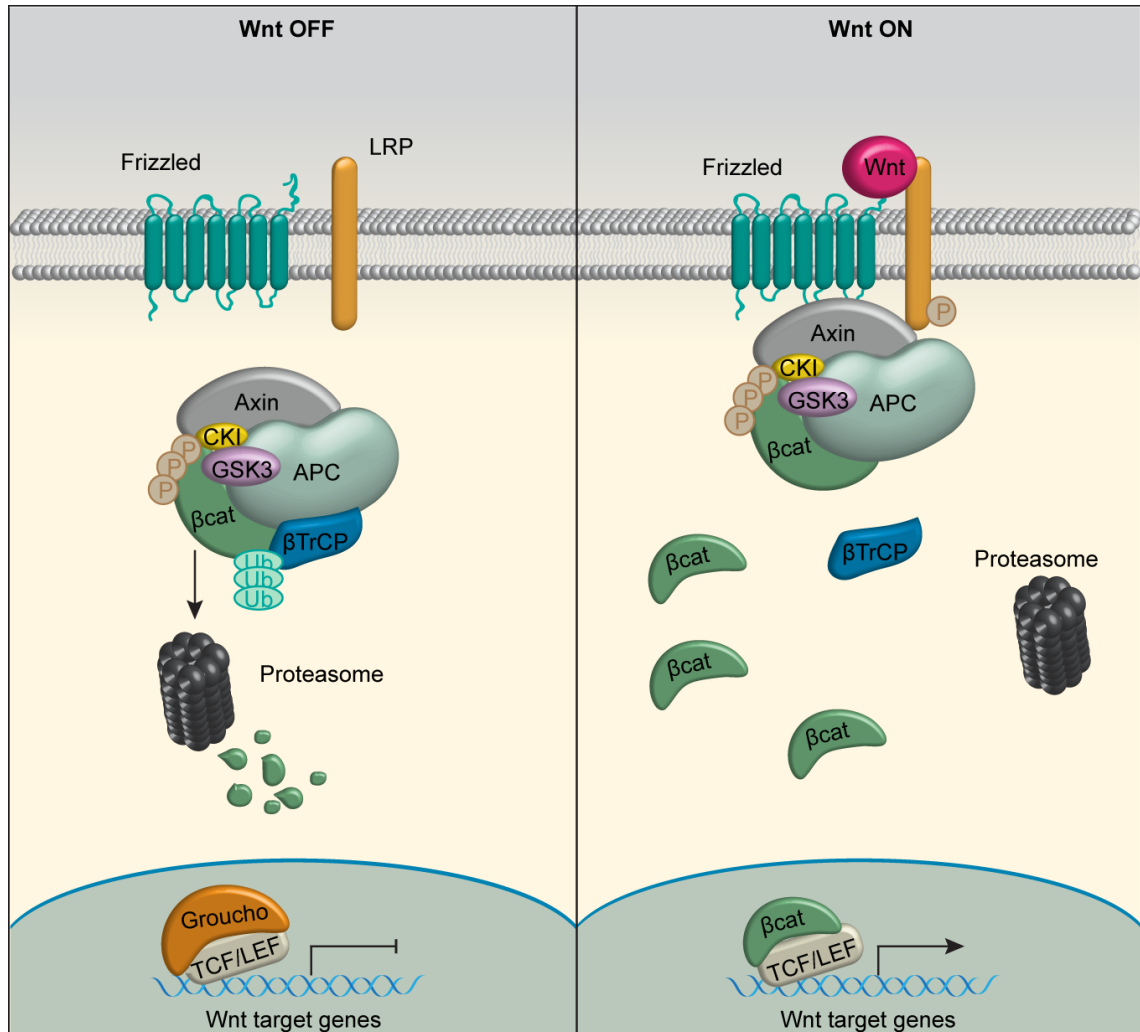


Figure 1.2 Schematic diagram showing overview of Wnt/β-catenin signalling pathway.

In the absence of Wnt (left panel), the Wnt/β-catenin transcription program is blocked due to the degradation of β-catenin by the cytoplasmic destruction complex. Wnt engagement with the receptors (right panel) activates Wnt signalling cascade by inhibiting the β-catenin destruction complex and initiates the Wnt transcription program.

1.2.3 Receptors and ligands for Wnt/β-catenin signalling

Wnt protein. The discovery of the Wnt protein was a fascinating scientific story which began in 1982 (Nusse and Varmus, 1982). The *Wnt* gene was initially named as *Int1* proto-oncogene (to denote the first common integration site), which

was later found to be homologous to the *Drosophila Wingless*. In the early 90s it was then renamed as Wnt1 (for Wingless-related integration site) (Nusse et al., 1991). As Nusse and Varmus wrote, *“to understand how those facts were unearthed and assembled into coherent concepts, it is necessary to probe the history of a field—to learn about the sequence of events, the logical and illogical connections between those events, and the people who participated in them”* (Nusse and Varmus, 2012).

Wnt proteins are members of a family of signalling molecules that are evolutionarily conserved (Holstein, 2012). In mammals, 19 independent genes encode for Wnt proteins. Wnt proteins are 40 kDa proteins rich in cysteines that are post-translationally modified by attachment of glycoproteins (glycosylation) and lipids, mainly palmitate and palmitoleic acid (acylation) (Rios-Esteves and Resh, 2013; Takada et al., 2006; Willert et al., 2003). Interestingly, Wnt glycosylation seems to be crucial for protein secretion but not for their signalling capability. For example, Wnt3a mutants lacking glycosylation results in impaired secretion (Komekado et al., 2007). On the other hand, purification of Wnt5 proteins *in vitro* that are deglycosylated, remain capable of activating intracellular signal cascades (Kurayoshi et al., 2007). The attachment of palmitoleic acid functions as a binding motif to the Wnt protein receptors (Janda et al., 2012) and is crucial for signalling activation (Komekado et al., 2007; Rios-Esteves et al., 2014; Willert et al., 2003). It also renders the Wnt protein hydrophobic, which may contribute to the restricted range of action of the protein (Nusse and Clevers, 2017). This modification is mediated by Porcupine in the endoplasmic reticulum (ER) (Rios-Esteves and Resh, 2013) at the conserved cysteine sites. After Wnt proteins are modified in the ER, they are transported and secreted by the multiple pass transmembrane protein Wntless/evensen's interrupted (Wls/Evi) (Nusse and Clevers, 2017). The intracellular trafficking complex Retromer is also fundamental for Wnt protein secretion (Coudreuse et al., 2006). One of the key functions of Retromer is the recycling of the Wls/Evi endosomal vesicles back to the trans-Golgi network (Port et al., 2008). In addition, vacuolar acidification and specific cargo proteins, such as the p24 family are required for Wls transport and release of Wnt protein (Buechling et al., 2011; Coombs et al., 2010). At the membrane, Wnt proteins are secreted via exocytosis to activate Wnt signalling in neighbouring cells. How extracellular signal is transferred to target cells remains unclear. Evidence suggests that Wnt proteins are incorporated into exosomes in such a form that Wnt ligand is presented in the outside membrane of the exosome

and presented to the receptors of the target cell (Gross et al., 2012; Korkut et al., 2009; Saha et al., 2016).

The existence of 19 Wnt proteins raises questions regarding their specificity. On one hand, deletion of different Wnts results in different phenotypes, supporting the idea of their distinct developmental roles (Chen et al., 2017; Farin et al., 2012; Stark et al., 1994). However, the high degree of conservation throughout evolution together with some *in vitro* experiments suggests that many Wnt proteins perform similar roles (Nusse and Clevers, 2017). It is generally believed that the signalling and functional specificity is driven by a combination of factors, including the local expression of individual Wnt proteins and the specific ligand-receptor interactions.

Wnt/FZD/LRP co-receptors complex. FZD proteins are seven-pass transmembrane receptors with an extracellular N-terminal cysteine-rich domain (CRD) (Bhanot et al., 1996). In humans, there are 10 FZD genes. As previously described, Wnt proteins interact with both FZD receptors and another single-pass transmembrane receptor LRP5/6 (Pinson et al., 2000; Tamai et al., 2000). The FZD-LRP co-receptor complex transduces Wnt signal by engaging with cytoplasmic proteins, particularly with DVL and AXIN. DVL proteins contain three main functional domains: DIX (Dishevelled-Axin); PDZ (postsynaptic density 95, disc large, zona occludens-1), and DEP (Dishevelled, Egl-10, Pleckstrin). The PDZ and DIX domains are essential for DVL to bind with FZD (Wong et al., 2003) and AXIN (Schwarz-Romond et al., 2007). Some studies have also suggested that DEP is important for DVL-FZD interaction (Simons et al., 2009; Tauriello et al., 2012; Wong et al., 2003). PDZ binds to the FZD KTxxxW motif (Umbhauer et al., 2000; Wong et al., 2003), thus facilitating the interaction between the AXIN and Wnt receptors. Wnt activation induces the LRP6 signalosome that aggregates receptors, DVL and AXIN to promote LRP6 phosphorylation (Bilić et al., 2007). AXIN recruitment to the membrane leads to the phosphorylation of the cytoplasmic LRP tail PPP(S/T)P motif by GSK3 and CK1 (Davidson et al., 2005; Zeng et al., 2005). It has been reported the DVL-AXIN interaction inhibits the β -catenin destruction complex by interfering with AXIN oligomerisation via the DIX domain (Fiedler et al., 2011). Furthermore, DVL polymerization is also believed to be essential for signalling activity (Schwarz-Romond et al., 2007). In addition, β -TrCP has been reported to dissociate from the

destruction complex when it is recruited to the membrane, resulting in suppression of β -catenin ubiquitination and Wnt signal activation (Li et al., 2012).

LGR5/R-spondin and Wnt signal regulation. The Wnt target gene and stem cell marker *Lgr5* and its homologues *Lgr4* and *Lgr6* have been shown to play crucial roles in mediating Wnt/ β -catenin signalling (de Lau et al., 2011; Glinka et al., 2011). *Lgr6* marks stem cells in the hair follicle (Snippert et al., 2010a). *Lgr4* and *Lgr5* genes are co-expressed in the ISC compartment. However, whilst *Lgr5* is exclusively expressed by ISCs, expression of *Lgr4* is detected throughout all crypt cells (de Lau et al., 2011). It is now clear that the orphan 7-transmembrane proteins LGR4/5/6 constitute receptors for the Wnt agonist R-spondin (RSPO) required for Wnt signal amplification (Carmon et al.; de Lau et al., 2011). Indeed, transgenic expression of RSPO1 has previously been shown to strongly promote hyperproliferation of intestinal crypt cells via Wnt/ β -catenin signalling activation (Kim et al., 2005). The transmembrane E3 ubiquitin ligases RING finger 43 (RNF43) and zinc and RING finger 3 (ZNRF3) have been recently reported to suppress Wnt/ β -catenin signalling by removing the FZD/LRP Wnt receptors from the cell surface (Hao et al., 2012; Koo et al., 2012) (see section Wnt/ β -catenin inhibitors). The association of RSPO with LGR4/5 receptors is believed to boost Wnt signalling by neutralising the Wnt negative feedback mechanism mediated by RNF43 and ZNRF3. The formation of the LGR/RSPO/RNF43-ZNRF3 complex prevents Wnt receptor internalization, thereby potentiating the Wnt signalling response (Hao et al., 2012; Lau et al., 2014).

Alternative Wnt signalling ligands and receptors. Apart from RSPO, the cysteine-knot protein, Norrin, has also been reported to bind to LGR4 (Deng et al., 2013) and FZD4 (Xu et al., 2004) to activate Wnt/ β -catenin signalling. On the other hand, the secreted Dickkopf (Dkk) proteins inhibit Wnt signalling by directly binding to LRP5/6 (Glinka et al., 1998). Binding of Dkk1 to LRP and the transmembrane Kremen proteins promotes Wnt receptor internalisation and signalling inactivation (Mao et al., 2002a).

Another transmembrane tyrosine kinase, Ryk receptor, has also been shown to bind to Wnt1 and Wnt3a and might function as a co-receptor along with FZD (Lu et al., 2004). The study also suggested that the association between FZD and DVL

may occur indirectly through Ryk to drive Wnt/ β -catenin signal activation. However, the underlying molecular mechanism of Ryk-mediated Wnt activation remains unknown.

As mention in section 1.2.1, the kinase receptors ROR family are also known to bind Wnt ligands (Green et al., 2014). ROR receptors are evolutionarily conserved across vertebrates and most of the species contain two homologs, ROR 1 and ROR2 (Green et al., 2014). The extracellular portion of the receptor contains a CRD domain with close homology to the FZDs and SFRPs CRD- domains (Jose Saldanha et al., 1998). It has been shown that Wnt5A is a ligand for ROR1 (Fukuda et al., 2008) and ROR2 (Oishi et al., 2003). Yamamoto and collaborators have shown that Wnt5A induces the formation of a complex between ROR2 and FZD, resulting in the recruitment of DVL, AXIN, and GSK3 β , the same proteins involved in the Wnt3A-induced phosphorylation of LRP5/6 (Yamamoto et al., 2007). Therefore, the identity of the Wnt ligand determines whether the FZD co-receptor will be LRP5/6 or ROR2, thus dictating whether β -catenin-dependent or -independent signalling is activated, respectively (Grumolato et al., 2010).

1.2.4 The β -catenin destruction complex

β -catenin is the central player in the Wnt/ β -catenin signalling to drive Wnt target gene transcription. Its intracellular concentration is closely regulated by the cytoplasmic destruction complex. The scaffold protein AXIN directly interacts with all components of the complex, including β -catenin, APC, DVL, and two kinase families (CK1 α , - δ , - ϵ , and GSK3 α and - β). CK1 α is the major isoform responsible for β -catenin phosphorylation (Liu et al., 2002). GSK3 α and - β appear to have redundant functions in the destruction complex (Doble et al., 2007). APC contains multiple 15 and 20 amino acid repeats that bind to β -catenin (Spink et al., 2001). Between the second and the third 20 amino acid repeats, there is another highly conserved regulatory domain, the β -catenin inhibitory domain (CID), which is believed to be essential in downregulating β -catenin levels (Kohler et al., 2009; Roberts et al., 2011). However, little is known about the underlying mechanism. CK1 and GSK3 sequentially phosphorylate β -catenin at a series of highly conserved Ser/Thr

residues near its N terminus. CK1 initially phosphorylates β -catenin at Ser45 (Amit et al., 2002; Liu et al., 2002), which primes the subsequent GSK3-mediated phosphorylation at Thr41, Ser37 and Ser33. Phosphorylated β -catenin is then recognised by the F box/WD repeat protein β -TrCP. As a consequence, β -catenin is ubiquitinated and targeted for rapid destruction via proteasomal degradation (Aberle et al., 1997).

In addition to β -catenin degradation, the stability of the scaffold protein AXIN is also known to be important for Wnt signalling. Wnt activation has previously been shown to promote AXIN degradation (Yamamoto et al., 1999). Recent studies have further identified two E3 ligases, RNF146 and SIAH1/2, that are responsible for AXIN degradation (Ji et al., 2017; Zhang et al., 2011). However, multiple studies have demonstrated that β -catenin stabilisation precedes AXIN degradation (Li et al., 2012; Liu et al., 2005; Yamamoto et al., 1999), suggesting that AXIN degradation might be important to sustain, rather than initiate, Wnt signal activation.

1.2.5 Wnt signalling in the nucleus

The Wnt-induced transcription programme is mediated by TCF/LEF binding to the WNT transcriptional enhancers, which requires the co-activator β -catenin. β -catenin interacts with an assembly of other proteins, collectively named the Wnt enhanceosome (Fiedler et al., 2015). Pygopus recruits β -catenin via the BCL9 adaptor chromatin reader to the enhanceosome (Fiedler et al., 2015; Flack et al., 2017). Together with other coactivators such as TATA-binding protein (TBP), Brahma-related gene-1 (BRG-1) (Barker et al., 2001), CREB-binding protein (CBP)/p300 (Cadigan and Waterman, 2012), Mediator subunit 12 (MED12) (Kim et al., 2006) and Hyrax/Parafibromin (Mosimann et al., 2006), the Wnt transcription program is activated. Recent studies have shown that β -catenin interaction with the Wnt enhanceosome induces conformational changes (Tienen et al., 2017), which enable ubiquitination of the co-repressors Groucho/transducing-like enhancer of signal transduction (Gro/TLE) by the ubiquitin ligase UBR5 (Flack et al., 2017). Ubiquitination of Gro/TLE also involves the ATPase valosin-containing protein (VCP/p97) that regulates the folding of various cellular substrates including the ubiquitylated Gro/TLE (Flack et al.,

2017). This modification of Gro/TLE destabilises the tetramer structure, thus relieving chromatin compaction to allow transcriptional activation. Conversely, silencing of the Wnt transcription program requires new chromatin compaction via the action of Gro/TLE on the SWI/SNF chromatin remodelling complex (Collins and Treisman, 2000; Sekiya and Zaret, 2007).

Functional studies have confirmed that the β -catenin/TCF/LEF transcription programme is essential for maintenance of the crypt progenitor compartments in the intestine. For example, deletion of the *Tcf4* transcription factor or conditional depletion of β -catenin in the intestinal epithelium results in loss of intestinal epithelial integrity (Ireland et al., 2004; Korinek et al., 1998). Vertebrates harbour four *Tcf/Lef* genes. All of them contain a highly conserved high-mobility group (HMG) box which has a DNA binding function and a peptide motif of basic residues (HMG DBD) that can increase the DNA binding (Klaus Giese et al., 1991; Love et al., 1995; Wetering and Clevers, 1992). TCF4 is the only isoform without HMG DBD (Kennell et al., 2003). Chromatin immunoprecipitation experiments have confirmed that the AGATCAAAGG sequence is the consensus TCF4/ β -catenin binding motif (Blahnik et al., 2010; Bottomly et al., 2010; Hatzis et al., 2008).

The process by which β -catenin enters the nucleus is still not fully understood. β -catenin itself has no nuclear localisation signal (NLS), whilst its import is believed to be via nuclear pore machinery such as importin/karyopherins (Fagotto et al., 1998). β -catenin can also be actively transported back to the cytoplasm, through either an intrinsic nuclear export signal or as cargo of Axin (Cong and Varmus, 2004) or APC (Rosin-Arbesfeld et al., 2000). The β -catenin shuttle between cytoplasm and nucleus seems to be a dynamic mechanism that may require upstream signalling regulation as another regulatory mechanism for this pathway.

1.2.6 Wnt/ β -catenin inhibitors

Biological signalling pathways are precisely regulated to control cellular processes such as proliferation, apoptosis and differentiation that are critical for tissue homeostasis. Wnt signalling is regulated at different levels by a wide range of intracellular and extracellular effectors. The presence of agonists and antagonists is

of extreme importance to control Wnt signalling at the correct level. Wnt agonists such as RSPO and Norrin were described earlier. In this section, I discuss several important antagonists of the Wnt pathway in detail. The link between deregulation of Wnt inhibitors and CRC will also be discussed in a later section.

Dickkopf proteins. As mentioned earlier, DKKs are secreted glycoproteins that act as antagonists in Wnt/ β -catenin signalling by directly binding to the receptors LRP5/6 (Glinka et al., 1998). In vertebrates, the DKK family comprises 4 members, DKK1-4. However, DKK3 appears to be a divergent member (Krupnika et al., 1999; Luke et al., 2003) and does not affect Wnt signalling (Mao and Niehrs, 2003; Mao et al., 2001). DKKs inhibit Wnt signalling by preventing the interaction of Wnt ligands with LRP receptors, thus disrupting the formation of the Wnt induced FZD-LRP complex. DKKs also bind to another class of receptors, Kremen1 and 2 (Krm1/2). A study from Mao *et al* in 2002 showed that DKK1 forms a ternary complex with Krm2 and LRP6, thus promoting the internalisation of LRP6 and its subsequent inactivation (Mao et al., 2002b).

WISE and SOST. WISE has been reported to be a context-dependent activator or inhibitor of the Wnt signalling pathway. Both secreted and intracellular WISE are able to inhibit Wnt signalling by competing with Wnt ligands to bind to LRP6 at the membrane level (Itasaki, 2003). Another close family, Sclerostin (SOST), has also been shown to antagonise Wnt signalling by binding to the first two YWTD-EGF repeat domains of LRP5/6 to disrupt the FZD-LRP co-receptor complex formation (Semënov et al., 2005). Interestingly, structural analysis revealed that WISE and SOST belong to a subfamily of cysteine knot BMP antagonists, although the precise mechanism of action is unclear (Avsian-Kretchmer and Hsueh, 2004).

WIF-1. Wnt-inhibitory factor 1 encodes a 379- amino acid protein that contains a WIF domain to prevent Wnt proteins from binding to its receptors, leading to Wnt signalling suppression. WIF-1 can bind to several Wnt proteins, including Wnt3A, Wnt4, Wnt5a, Wnt7a, Wnt9a and Wnt 11 to regulate Wnt activity(Surmann-Schmitt et al., 2009).

SFRPs. The secreted frizzled-related proteins comprise a family of five members, SFRP1-5 in humans. SFRP1, -2, and -5 form a subgroup that diverges from SFRP3 and -4 (Cruciat and Niehrs, 2013; Jones and Jomary, 2002). These proteins contain a CRD domain at the amino-terminal similar to the FZD receptors (Hoang et al., 1996), and a netrin-related motif (NTR) at the carboxy-terminal (Ramesh A. Bhat et al., 2007). Both CRD and NTR domains appear to be important for Wnt ligand binding and signalling inhibition (Wawrzaka et al., 2007). Another alternative mechanism for SFRP-mediated Wnt inhibition could be via formation of non-functional complexes with FZD receptors, since CRDs have dimerization potential (Cruciat and Niehrs, 2013; Dann et al., 2001).

1.2.7 Wnt/ β -catenin negative feedback loop

Apart from the Wnt inhibitors summarised in the previous section, there are several other Wnt inhibitors that belong to the negative feedback loop of the Wnt/ β -catenin signalling. These inhibitors are transcriptionally regulated by the β -catenin/TCF complex and function as a negative feedback mechanism to control Wnt signalling at various levels of the cascade. Being Wnt transcriptional targets, these negative feedback Wnt inhibitors are often expressed at the bottom of intestinal crypts where Wnt activity is high.

Notum. Notum encodes a protein of 671 amino acid residues that are related to plant pectin acetylsterases of the α/β -hydrolase superfamily. This sequence similarity suggests that Notum could hydrolyse glycosaminoglycan chains of glypicans (Doerks et al., 2002; Giráldez et al., 2002), therefore removing the post-translational modifications of the Wnt ligands, and affecting its capacity for binding Wnt receptors. More recently, Kakugawa and colleagues revealed that Notum is indeed an extracellular deacylase that removes the palmitoleate moiety from Wnt proteins (Kakugawa et al., 2015). It has been reported that Notum expression is induced by Wnt signalling, thus placing Notum in the category of negative feedback Wnt inhibitors (Giráldez et al., 2002; Torisu et al., 2008)

RNF43 and ZNRF3. As previously discussed, RNF43 and ZNRF3 are capable of removing the FZD/LRP Wnt receptors from the cell surface, thus inhibiting the sensitivity to Wnt stimulation (Hao et al., 2012; Koo et al., 2012). Biochemical analyses further showed that RNF43/ZNRF3-mediated Wnt receptor endocytosis could be reversed by RSPO, suggesting that RSPO potentiates Wnt signalling by inhibiting RNF43/ZNRF3 (Hao et al., 2012). RNF43 has also been proposed as a Wnt transcriptional target, indicating that RNF43/ZNRF3 is expressed upon Wnt induction to regulate Wnt/ β -catenin signalling via a negative feedback mechanism (Koo et al., 2012; Takahashi et al., 2014).

AXIN2. As described earlier, AXIN is the key scaffold protein in the cytoplasmic destruction complex that negatively regulates Wnt signalling by mediating β -catenin degradation (Behrens et al., 1998; Yamamoto et al., 1998). There are two AXIN homologues, AXIN1 and AXIN2 (also known as Conductin), that are believed to function redundantly. On the other hand, only AXIN2 is reported as a direct Wnt transcriptional target (Lustig et al., 2002), indicating its unique negative feedback role in Wnt/ β -catenin signalling control.

SOX9. It has been proposed that SOX9 inhibits Wnt signalling by competing with TCF/LEF for binding to β -catenin to control chondrocytes fate determination (Akiyama et al., 2004). In the intestine, Sox9 has been reported as a direct Wnt transcriptional target that is expressed at the Wnt-active crypt compartment to repress differentiation (Blache et al., 2004). Deletion of Sox9 in the gut results in loss of Paneth cells, upregulated Wnt target gene expression and intestinal hyperplasia (Bastide et al., 2007; Mori–Akiyama et al., 2007). The mechanism by which SOX9 inhibits Wnt/ β -catenin is not yet fully understood. In addition to the possible physical competition with TCF for β -catenin, Sox9 has also been shown to promote β -catenin phosphorylation in the nucleus and its subsequent degradation (Topol et al., 2009).

1.3 Notch signalling

Intestinal homeostasis requires the correct balance between self-renewal, cell death and differentiation. In addition to Wnt signalling, multiple other pathways are also involved in regulating intestinal homeostasis. In the stem cell niche, activation of both Wnt and Notch signals are fundamental for stem cell maintenance (Korinek et al., 1998; van Es et al., 2005). Notch signal transduction is initiated when the membrane-bound Notch ligand (Delta/Jag) binds to the Notch receptor of a neighbouring cell (Figure 1.3). This results in cleavage of the receptor by the γ -secretase protease complex to release the Notch intracellular domain (NICD). The released NICD translocates into the nucleus, where it binds to the recombining binding protein suppressor of hairless (RBPJ-K) to activate Notch target gene transcription (Tamura et al., 1995). Activation of the Notch transcription programme results in the expression of the Hairy and Enhancer of Split (Hes) class genes that repress expression of the Notch ligand, Delta. Thus, activation of Notch signalling represses the production of the Delta ligand, resulting in an important lateral inhibition process. A cell with lower Notch activity produces more ligand, which subsequently activates Notch signalling in the neighbouring signal-receiving cell. In the case of the intestine, Notch signalling is key to drive both stemness and lineage specification. Paneth cells have been shown to express Notch ligand to activate Notch signalling in the ISCs (Sato et al., 2011). In the TA compartment, Notch high progenitors will differentiate to absorptive enterocytes, whilst the neighbouring progenitor cells with low Notch activity will commit to the secretory fate (Sancho et al., 2015).

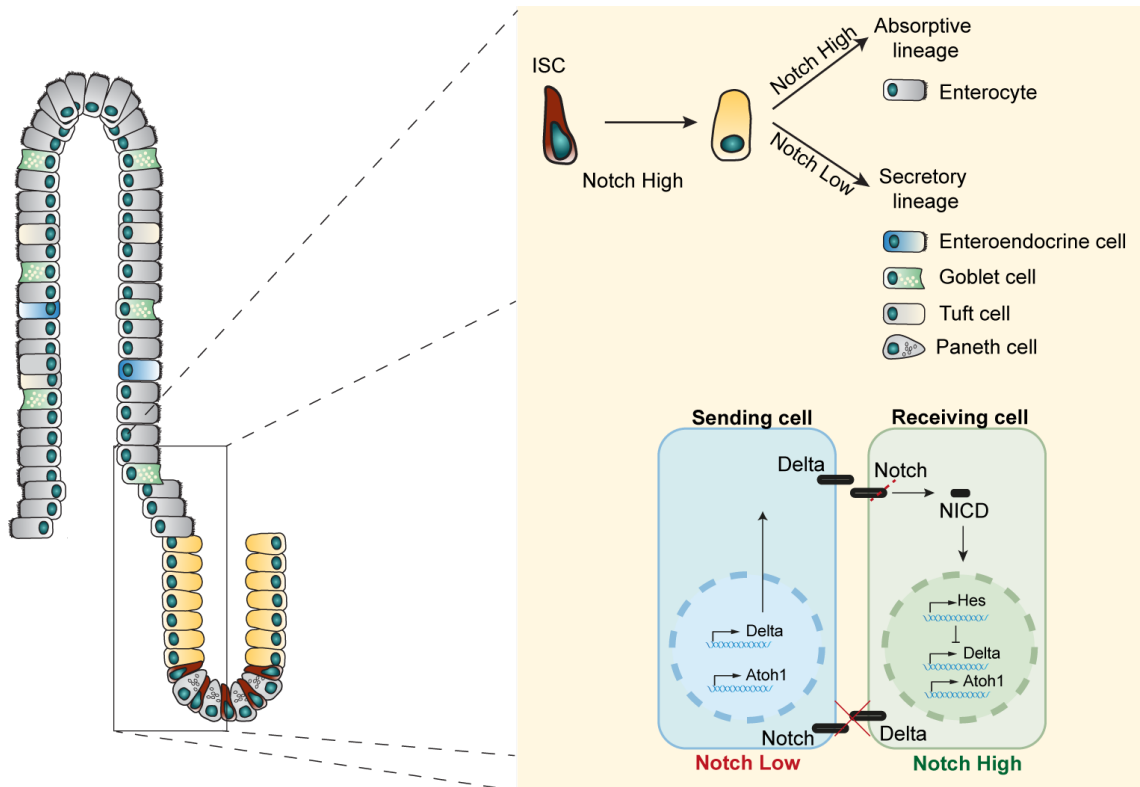


Figure 1.3 Intestinal homeostasis and Notch signalling.

Notch signalling plays a crucial role in intestinal stem cell and progenitors differentiation. ISC's pool is maintained by constant Notch ligand provided by Paneth cells. In the TA compartment, Notch acts to promote differentiation. Notch-high earlier progenitors will differentiate to enterocytes, whilst Notch-low neighbouring cells commit to a secretory fate (enteroendocrine cells, goblet cells, Tuft cells and Paneth cells). Notch signal is initiated when a cell-surface-expressed Delta ligand binds to Notch receptor expressed on the neighbour cell. The membrane-tethered Notch is cleaved by ADAM10 and γ -secretase complex to release the intracellular fragment of Notch (NICD). NICD translocates to the nucleus and binds to a transcriptional activation complex that relieves repression of Notch target genes such as Hes family. The Hes family of transcriptional repressors reduces the levels of the Notch ligand Delta, thus resulting in a feedback loop between the two neighbouring cells, known as lateral inhibition - the cell with lower Notch activity produces Delta ligand, due to derepression of the transcription factor Atoh1. This allows the cell to constantly present the ligand to the neighbouring cell and activate Notch. This activation reduces the Delta ligand production which allow the cell with lower Notch activity to increase its ligand production even further since it keeps receiving low signal back. Therefore, the difference in Notch activity between adjacent cells is amplified promoting fate specification into different lineages.

1.4 EGFR signalling

The EGF receptor tyrosine kinase (EGFR) signalling cooperates with other pathways such as Wnt/ β -catenin to promote normal tissue development, whilst the crosstalk of the pathways appears to be context-dependent. For example, in *Drosophila* eye, it has been shown that EGFR signalling antagonizes Wnt signalling (Freeman and Bienz, 2001) and the EGFR transcriptional target Phyllopod blocks *Wingless* (Nagaraj and Banerjee, 2009). On the other hand, both pathways act together to induce male hook development in *C.elegans* (Yu et al., 2009). Different crosstalk mechanisms have been proposed between Wnt/ β -catenin and EGFR signalling. These include transactivation of EGFR upon Wnt binding to FZD receptors (Civenni et al., 2003), direct activation of β -catenin signalling by EGFR through Ras/MAPKs signalling (Yang et al., 2011) and modulation of the cadherin- β -catenin binding via EGFR-mediated β -catenin phosphorylation (Lilien and Balsamo, 2005).

In the intestine, EGFR signals are thought to be important for ISC maintenance and cell differentiation (Sato et al., 2011) (Figure 1.4). The EGF-mediated proliferation of intestinal epithelial cells is tightly regulated by the expression of the EGFR inhibitor *Lrig1* (Wong et al., 2012), where its genetic inactivation results in rapid expansion of the proliferative compartment (Powell et al., 2012; Wong et al., 2012). Ras is activated through GTP-loading by Ras guanine nucleotide exchange factors (RasGEFs). A study from Depeille et al. has shown that Ras activation through different RasGEFs can result in different outputs (Depeille et al., 2015). Two distinct RasGEFs, RasGRP1 and SOS1, act functionally opposite to one another (Depeille et al., 2015). RasGRP1 restricts EGFR-SOS1-Ras signalling via a negative feedback mechanism. Increased crypt proliferation and goblet cell numbers were also observed upon RasGRP1 deletion (Depeille et al., 2015), suggesting that the level of EGFR-Ras signalling is crucial for intestinal stem cell fate control. A recent study further demonstrated the cooperative role of EGF, Wnt and Notch signalling in regulating enteroendocrine cell differentiation in the intestine (Basak et al., 2016). Taken together, the data indicate the essential role of EGF signalling in intestinal homeostasis via crosstalk with the Wnt pathway.

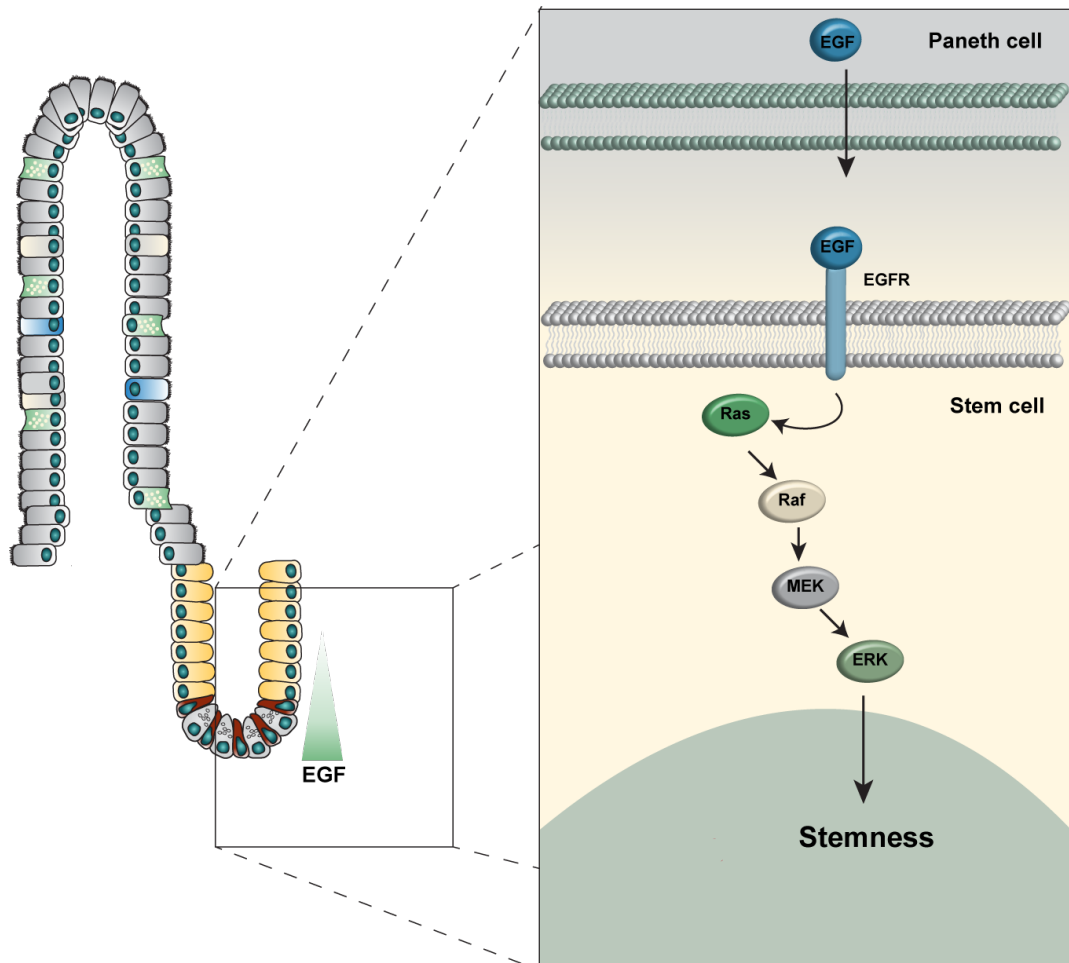


Figure 1.4 EGFR signalling in intestine.

EGFR is required for intestinal epithelial “stemness”. It is active at the bottom of the crypt in a gradient manner similar to Wnt signalling. Paneth cells constitute the niche for ISC by providing growth factors like EGF. Upon engagement of the EGFR, EGF initiates the RAS/ERK MAP kinase signalling activating a transcriptional program that promotes stem cell migration, proliferation, and inhibits apoptosis.

1.5 BMP signalling

Bone morphogenic proteins (BMPs), are extracellular signalling cytokines belonging to the large transforming growth factor-beta (TGF- β) superfamily. BMPs are known to play crucial roles in many organ systems. To date, more than 20 BMP ligands have been identified in humans. The BMP ligand initiates signalling by binding to and bringing together BMPRI and BMPRII receptors (Rahman et al., 2015). The intracellular proteins SMADs (mothers against decapentaplegic) are fundamental for signal transduction. These proteins transduce the signal from membrane to nucleus through receptor-mediated phosphorylation of SMAD transcription factors (Massagué et al., 2005). Eight SMAD proteins are encoded in human and mouse genomes. SMAD1/2/3/5 and 8 signal through the TGF- β receptor and are commonly known as regulated SMADs (R-Smads). SMAD6 and 7 are inhibitory SMADS that can interfere with receptors or with SMAD–SMAD interactions. SMAD4, also known as C-SMAD, serves as a common partner for all R-Smads (Rahman et al., 2015).

In mouse intestine, *Bmp4* appears to be exclusively expressed in the mesenchyme surrounding the villi (He et al., 2004). BMP signalling functions as a negative regulator of crypt progenitors. Conditional deletion of the *Bmpr1a* receptor results in hyperproliferative crypt epithelial cells (He et al., 2004), whilst overexpression of the BMP inhibitor Noggin and Gremlin results in ectopic crypt formation in the villi (Davis et al., 2015; Haramis et al., 2004). A recent study further demonstrated that BMP signalling restricts ISC stemness by direct SMAD-mediated repression of ISC signature genes (Qi et al., 2017). Mutations of BMP components, including *SMAD4* and *BMPRI1A* mutations, have been reported in different types of CRCs and in a subset of juvenile polyposis syndrome (Howe et al., 2001; Howe et al., 1998). Similarly, *Gremlin1* duplication has been reported in hereditary mixed polyposis syndrome patients (Jaeger et al., 2012). This highlights the crucial role of BMP signalling in both intestinal homeostasis and CRC development.

1.6 Colorectal cancer biology

CRC is a leading cause of cancer-related death in developed countries (Ferlay et al., 2013; Siegel et al., 2017; WHO, 2014). Over the past decades, genetic studies have revealed critical mutations underlying CRC. Loss of *adenomatous polyposis coli* (APC) function and subsequent mutations in other tumour suppressors or oncogenes such as *TP53*, *SMAD4*, *KRAS* and *PI3K* are required for cancer progression (Fearon, 2011; Sjoblom et al., 2006). Therefore, CRC is a result of multistep accumulation of genetic events known as “the Vogelgram sequence” (Fearon and Vogelstein, 1990), in which the number of mutations increases from the early adenomatous lesions to carcinomas and metastatic tumours.

Large genomic studies of CRC samples have revealed a wide range of possible players in CRC. In addition, events such as epigenetic silencing, crosstalk between different signalling pathways, intertumour heterogeneity across individual CRC patients, intratumour heterogeneity, tumour microenvironment and gut microbiota, add further complexity and challenges to the understanding of CRC initiation and progression.

Patients diagnosed with CRC are broadly divided into two major groups based on their molecular profile: microsatellite instability (MSI) or microsatellite stable (MSS). MSS-CRCs constitute more than 80% of CRCs. Loss of function of APC protein and low prevalence of somatic copy number alterations (SCNAs) are the hallmark of this group of patients (TCGA, 2012). On the other hand, the molecular fingerprint of patients with MSI tumours is the presence of insertions or deletions of nucleotides in microsatellite repeat regions, widespread in the entire genome, due to the defective DNA mismatch repair machinery. For this reason, MSI-CRCs are featured by hypermutator and hypermethylated phenotype.

Wnt pathway is aberrantly upregulated in both MSS and MSI-tumours, however in the MSI-CRCs, mutations in key components of the Wnt pathway, such as *APC*, *β -catenin* and *Axin2* are only observed in a subpopulation of MSI-CRCs (TCGA, 2012), suggesting an alternative Wnt activating mechanism for this tumour type. MSI-CRCs patients have worse overall survival and worse relapse-free survival (De Sousa et al., 2013; Guinney et al., 2015). This type of tumours shows upregulation of genes associated with matrix remodelling, epithelial-mesenchymal

transition and immune infiltrate, mainly composed of TH1 and cytotoxic T cells, along with strong activation of immune evasion pathways, which could potentially explain the worse prognosis (De Sousa et al., 2013; Guinney et al., 2015; Llosa et al., 2015).

In this section, I will discuss CRC biology with a particular focus on the Wnt activating mechanism in CRCs, starting with the most common mutated gene in CRC - *APC*.

1.6.1 *APC* mutations in colorectal cancer

***APC* germ-line mutations.** Mutations in *APC* is the cause of the familial adenomatous polyposis (FAP) syndromes. These syndromes are characterised by the early onset of multiple adenomatous polyps throughout the colon and rectum. Although a few adenomas progress to CRC, these patients have an increased predisposition to CRC development during lifetime (Fearon, 2011).

The germ-line mutations are distributed throughout the 5' region of the gene and the majority are frameshift mutations resulting in premature truncation of the protein. Interestingly, mutations in particular regions of the gene are associated with different polyposis status. Mutations between codons 1250 and 1464 are associated with more profuse polyposis whereas mutations at N-terminal or C-terminal show more attenuated phenotype (Galiatsatos and Foulkes, 2006). Biallelic loss of *APC* in FAP patients is achieved through either loss-of-heterozygosity or additional somatic mutations as second hit. Some patients show combination of FAP syndromes with other tumours, such as brain tumours, known as Turcot syndrome, or epidermoid cysts and osteomas, known as Gardner syndrome (Galiatsatos and Foulkes, 2006).

***APC* somatic mutations.** *APC* mutations are found in approximately 80% of sporadic CRCs. These mutations are frequently clustered between codons 1309 and 1450, which is known as the mutation cluster region (Fearon, 2011). Similar to the germ-line mutations, the majority of sporadic *APC* mutations are frameshift and nonsense mutations that cause premature protein truncation. *APC* protein truncation activates Wnt/ β -catenin signalling through abrogation of the normal β -catenin destruction complex (Korinek et al., 1997). This aberrant activation of Wnt/ β -catenin signalling, and the accumulation of further mutations in other genes, results in the progression from adenomas to carcinomas.

1.6.2 CRC molecular subtypes

As mentioned above, CRCs have been previously broadly classified into two groups – MSS and MSI - based on their hypermutation profiles (TCGA, 2012). More recently, Guinney et al. performed a comprehensive cross-comparison of various genomic and transcriptomic studies of large collections of CRC cohorts, leading to an improved molecular classification system of CRC into four consensus molecular subtypes (CMSs) (Guinney et al., 2015). CMS1 group constitutes approximately 15% of all CRCs featuring the typical hypermutated MSI phenotype. These tumours are characterised by hypermethylation status – in particular, *MLH1* hypermethylation resulting in defective DNA repair machinery (Ionov et al., 1993; Thibodeau et al., 1993), low prevalence of somatic copy number alterations (SCNAs) and extensive immune infiltration (Llosa et al., 2015), mainly cytotoxic T lymphocytes, T helper 1 cells and natural killer cells (Guinney et al., 2015). Tumours with chromosomal instability are more heterogeneous at the gene-expression level and they can be subdivided into three groups: CMS2, 3 and 4. Tumour development of CMS2-4 generally follows the model proposed by Vogelstein (Fearon and Vogelstein, 1990) (Figure 1.5). CMS2 tumours are SCNA-high with approximately 80% of the cases having *APC* mutations, therefore displaying strong upregulation of WNT transcriptional targets. CMS3 tumours harbour high prevalence of *APC* and *KRAS* mutations but low SCNAs and intermediate levels of hypermethylation. Enrichment of several metabolic components is also observed in CMS3, suggesting a metabolic adaptation in this tumour subtype. Finally, CMS4 tumours display upregulation of genes involved in epithelial-to-mesenchymal transition, TGF- β signalling activation, angiogenesis and stromal infiltration (Guinney et al., 2015). This subtype is also characterised by poor disease outcome.

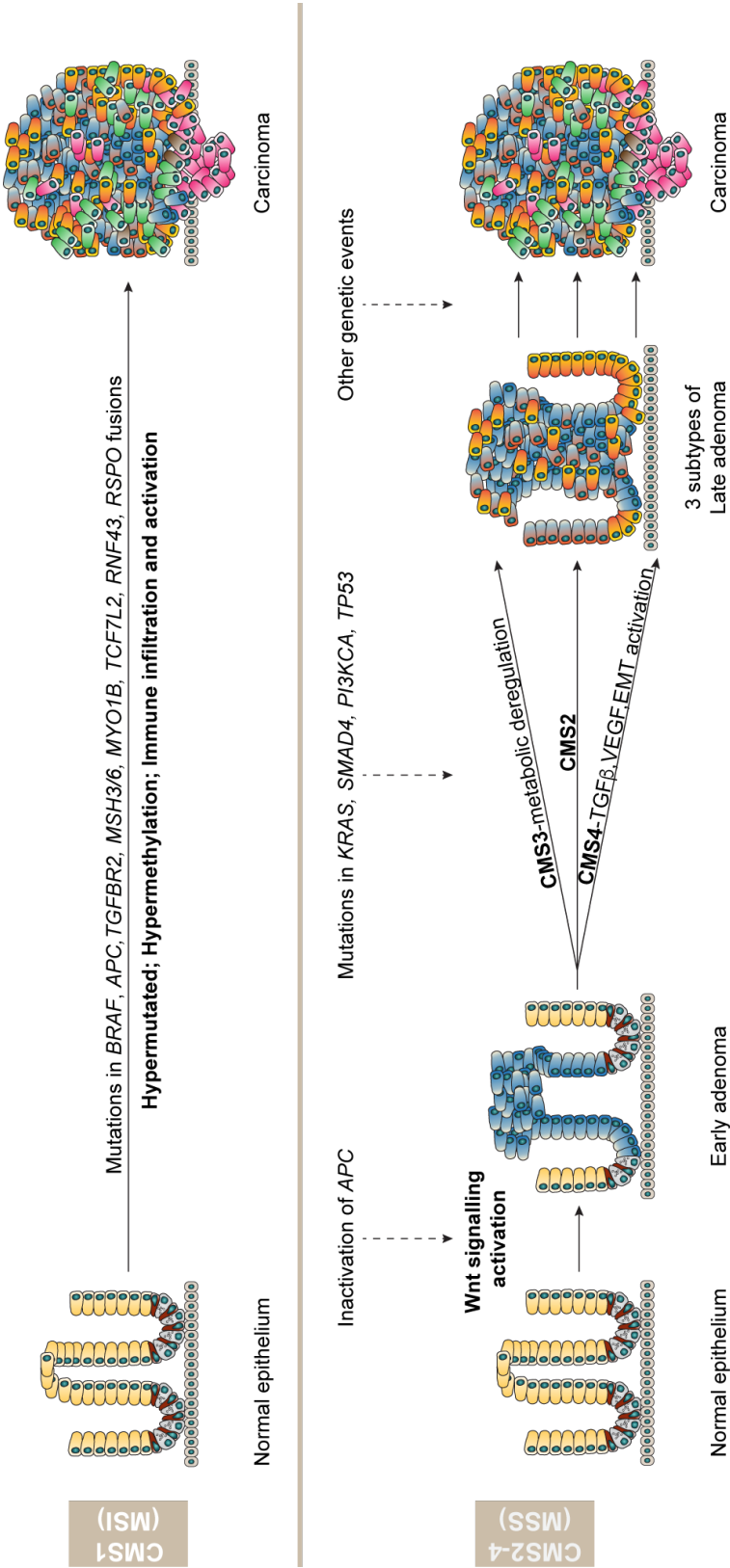


Figure 1.5 Molecular subtypes of colorectal cancer.

Simplified schematic diagram of the histopathological changes aligned with genetic abnormalities observed in different subtypes of CRC. Tumours with microsatellite instability (MSI – CMS1) are characterised by hypermutations, hypermethylation and immune infiltration. The most commonly observed mutations in CMS1/MSI tumours are highlighted, especially the frequent occurrence of *BRAF* mutations (Guinney et al., 2015; Rajagopalan et al., 2002). CMS1 CRCs carry *APC* mutations in less frequency than those described in CMS2-4, suggesting involvement of different genetic changes (such as *RNF43* and *RSPO*) but analogous pathological transformation. Tumours with microsatellite stable, chromosomal unstable (MSS – CMS2-4), follow the Vogelgram sequence. Adenoma formation is initiated with *APC* loss. Progressive step-wise accumulation of additional mutations such as *KRAS*, *SMAD4* and *TP53* results in progression from early adenoma to carcinoma. CMS3 is characterised by high frequency of *KRAS* mutations and is associated with multiple metabolism signatures. CMS2 and 4 cannot be distinguished by their mutation spectrum. However, CMS2 CRCs show upregulation of Wnt signalling pathways, whereas CMS4 tumours are characterised by activation of TGF β , EMT and VEGF signalling activation.

1.6.3 Cells of origin of CRC

The identification of various ISC markers subsequently led to the discovery of CRC origin. Barker et al. demonstrated that *Apc* deletion in *Lgr5*⁺ cells resulted in early adenoma transformation and progressive neoplasia (Barker et al., 2009). In a complementary manner, deletion of *Apc* in non-stem cells compartment, particularly TA cells, only led to small lesions that do not form tumours (Barker et al., 2009). A recent study further showed that *Lgr5*⁺ cells are indeed cancer-initiating cells that are required for metastasis (de Sousa e Melo et al., 2017). However, although ablation of *Lgr5*⁺ cells showed restriction of primary tumour growth, tumour regression was not observed. The *Lgr5*⁺ cells are believed to continuously proliferate and replenish the *Lgr5*⁺ cancer stem cell pool (de Sousa e Melo et al., 2017).

Several studies have also shown the presence of other normal stem cell markers such as CD133⁺, CD44⁺ and EpCAM30^{high} in a subpopulation of CRC tissues that are able to initiate tumour formation in mice, suggesting that these markers may also be cancer stem cell markers (Dalerba et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Snippert et al., 2009; Zhu et al., 2008). In addition, *EphB2*⁺ cells – which is highly expressed in ISCs - have also been shown to have tumour-initiating capacity (Merlos-Suarez et al., 2011).

In contrast to the studies showing stem cells as the cells of origin of CRC, there is some evidence supporting the notion of CRC developing via non-stem cells in mouse. For example, deletion of *Apc* in the differentiated *Dclk1*⁺ tuft cells does not result in tumourigenesis (Nakanishi et al., 2013). However, if these cells are challenged with dextra sulphate sodium to induce inflammation, they are capable of originating tumours (Westphalen et al., 2014). In another study, it has been shown that β -catenin mutations in differentiated cells do not result in transformed epithelium (Schwitalla et al., 2013). Yet, if mutations in β -catenin happen together with activation of NF κ B pathway, tumours are formed. This same study has also showed that activation of the NF κ B pathway result in crypt-like structures in the villus (Schwitalla et al., 2013). Davis and colleagues have also shown that a mouse model with aberrant activation of the BMP antagonist GREM1 resulted in the formation of crypt-

like structures in the villi, similar to was observed upon NF κ B activation (Davis et al., 2015). The accumulation of additional mutation in these crypt-like structures also resulted in intestinal tumours (Davis et al., 2015).

Altogether, these studies suggest that Wnt/ β -catenin signalling activation alone in non-stem cells is not sufficient to drive adenoma formation. However, if cells acquire additional mutations or if changes happen in the normal intestinal microenvironment, it could then result in tumour formation.

1.6.4 Mutations in Wnt signalling components in CRC

Aberrant Wnt activation is the hallmark of CRCs in both MSS (mostly CMS2-3) and MSI-tumours (CMS1) compared with normal tissues. Mutations in multiple key components of the Wnt pathway are found in the majority of CRCs (TCGA, 2012). More than 80% of MSS CRCs harbour *APC* mutations, as compared with 50% in MSI cases. On the other hand, epigenetic silencing of Wnt inhibitors in MSI tumours has been proposed as an alternative Wnt activating mechanism. Several studies have reported hypermethylation of extracellular Wnt inhibitors (Qi et al., 2006; Rawson et al., 2011; Silva et al., 2014; Suzuki et al., 2004; Taniguchi et al., 2005; Voorham et al., 2013) and of key scaffold proteins of the destruction complex (Esteller et al., 2000; Fu et al., 2012; Koinuma et al., 2006; Murakami et al., 2014). Recent studies have further identified genetic alterations in other Wnt pathway components (such as *RNF43* and *RSPO*) in MSI tumours required for Wnt signal activation (Giannakis et al., 2014; Seshagiri et al., 2012). In this section, and in addition to *APC* mutations mentioned above, I will summarise the mutations in Wnt pathway components commonly found in different CRC subtypes.

AXIN. Mutations of the negative Wnt regulators *AXIN1* and *AXIN2* are found in both sporadic and familial CRCs (Salahshor and Woodgett, 2005). Germline mutations in exon 7 (Lammi et al., 2004; Marvin et al., 2011) and exon 5 (Rivera et al., 2014) of *AXIN2* have been reported with genetic predisposition to CRC and tooth agenesis. Several studies have described somatic mutations of *AXIN2* in MSI CRCs, the majority of which are also localised in exon 7 (Liu et al., 2000b; Thorstensen et

al., 2005). In addition, epigenetic silencing of *AXIN2* has also been reported in MSI CRCs (Koinuma et al., 2006). Although no germline mutation of *AXIN1* has been identified in CRCs, several studies have reported *AXIN1* somatic mutations (Jin et al., 2003; Shimizu et al., 2002). Similar to *APC* mutation, loss of AXIN disrupts the β -catenin destruction complex function, resulting in constitutive Wnt/ β -catenin activation. A recent study has further shown that AXIN mutants form non-amyloid nanometer-scale aggregates that affect its normal interaction, and therefore the function of the β -catenin destruction complex (Anvarian et al., 2016).

WTX. WTX (also known as FAM123B or adenomatous polyposis coli membrane recruitment 1, AMER1), is an X-linked gene mutated in Wilms tumours. Previous studies suggest that WTX plays a dual role in Wnt signalling. On one hand, WTX interacts with the destruction complex to promote β -catenin ubiquitination and degradation (Major et al., 2007). On the other hand, WTX seems to promote Wnt-induced LRP6 phosphorylation (Tanneberger et al., 2011). In patients, truncating mutations of *WTX* are frequently observed in kidney and CRCs. More recent sequencing data revealed high prevalence of *WTX* mutations in MSI CRCs (TCGA, 2012). Further characterisation of the role of WTX in the Wnt signalling pathway will shed light on its clinical significance.

RNF43. Recent whole-exome sequencing of a large cohort of human CRCs identified a large number of non-silent somatic mutations in *RNF43* predominantly associated with MSI cases (Giannakis et al., 2014). As mentioned above, RNF43 and ZNRF3 are expressed at the bottom of intestinal crypts to negatively regulate Wnt signalling by controlling Wnt receptor endocytosis (Hao et al., 2012; Koo et al., 2012). Loss of function mutations in these proteins leads to increased sensitivity to the Wnt ligands and subsequent Wnt/ β -catenin signalling activation. Therefore, the Wnt activating mechanism of this CRC subtype is dependent on exogenous Wnt signal, indicating that drugs targeting Wnt ligand/receptor (such as porcupine inhibitor) could represent an ideal therapeutic strategy.

CTNNB1. *CTNNB1* (β -catenin) is the key mediator of Wnt signalling pathway. As previously described, phosphorylation at ser45 by CK1, followed by GSK3-

mediated phosphorylation at ser33, ser37, and thr41 are critical for β -catenin degradation (Liu et al., 2002). Mutations at these amino acid residues will result in β -catenin stabilisation and activation of Wnt signalling. Indeed, hotspot mutations at these NH2-terminal serine/threonine residues of *CTNNB1* such as S45F and T41A are frequently found in CRC, particularly in the MSI subtype (Morin et al., 1997). β -catenin is also a subunit of cadherin protein complexes that play essential roles in coordinating cell-cell adhesion. A recent study has suggested that E-cadherin can act as a buffer to sequester mutated β -catenin, thus limiting the transforming properties of activating β -catenin mutations (Huels et al., 2015).

RSPO. As discussed earlier, RSPO proteins are potent Wnt agonists that enhance Wnt signalling via engagement with the LGR and FZD receptors in the presence of Wnt ligands (de Lau et al., 2011; Glinka et al., 2011). Overexpression of RSPO1 in mice results in hyperproliferation and expansion of the intestinal crypts (Kim et al., 2005). Two recurrent gene fusions have recently been discovered in CRCs, *RSPO2-EIF3E* (eukaryotic translation initiation factor 3) and *RSPO3-PTPRK* (receptor-type tyrosine- protein phosphatase) (Seshagiri et al., 2012). Interestingly, these fusions were found in non-APC-mutated CRCs, indicating an alternative Wnt-activating mechanism.

TCF. TCF family proteins, including TCF7 (also known as TCF1), LEF, TCF7L1 (TCF3) and TCF7L2 (TCF4), are the downstream effectors of Wnt/ β -catenin signalling. Point mutations, deletions, and translocations of *TCF7L1* and *TCF7L2* have been reported in CRCs (Bass et al., 2011; Cuilliere-Dartigues et al., 2006; Duval et al., 1999; TCGA, 2012), whilst their Wnt-activating potential remains incompletely understood. Specific *TCF7L2* frameshift mutations identified in MSI CRCs have been shown to be activating mutations via the selective loss of the *TCF7L2* isoform with binding ability to the transcriptional repressor CtBP (Cuilliere-Dartigues et al., 2006). In mouse intestine, *Tcf4* deletion results in crypt degeneration, whereas loss of *Tcf1* and *Tcf3* do not result in any apparent defect (van Es et al., 2012a). Further investigation is required to explain the discrepancy between the impact of mutations between different TCF members and to characterise transcriptional regulation mediated by TCF1, TCF3, and TCF4.

1.6.5 Crosstalk between Wnt/ β -catenin signalling and other pathways in CRC

Signal transduction pathways often function in complex signalling networks that require high levels of regulation for precise control of cellular processes. In CRC, crosstalk of Wnt/ β -catenin signalling with other pathways adds further complexity to CRC biology. Here, I describe some examples of pathway crosstalk reported in CRC.

EGFR. As described above, EGF signalling cooperates with Wnt signalling to regulate proliferation and differentiation of stem and TA cells. Therefore, deregulation of the EGFR signal machinery can potentiate CRC initiation and progression. For example, deletion of RasGRP1 results in increased proliferation of intestinal epithelial cells (Depeille et al., 2015). In fact, *APC*-mutated adenomas in mice have been shown to be associated with increased EGFR activity (Moran et al., 2004). In addition, EGFR signalling has been reported to modulate cadherin- β -catenin interaction via phosphorylation of β -catenin Y654 (Lilien and Balsamo, 2005). Accordingly, expression of the phospho-mimicking Y654E β -catenin mutant in *Apc*-driven mouse model resulted in increased intestinal tumour frequency via enhanced Wnt signalling (van Veelen et al., 2011). The data suggest that EGFR and Wnt signalling function synergistically to promote intestinal tumour initiation and progression.

Notch. Crosstalk of Notch and Wnt signalling is crucial not only for normal intestinal homeostasis, but also for intestinal tumourigenesis. It has been reported that Notch signalling is activated downstream of Wnt/ β -catenin via both induced expression of Notch ligand/receptor and β -catenin-mediated Hes1 transcription (Peignon et al., 2011). Synergistic activation of both pathways in mice results in a dramatic increase in colonic tumours, which is uncommon in mouse models with only *Apc* mutation (Fre et al., 2009). On the other hand, inactivation of Notch signalling using γ -secretase inhibitors suppresses proliferation in *Apc*-driven intestinal tumours by inducing post-mitotic goblet cell differentiation (van Es et al., 2005). However,

several other studies have proposed a negative regulatory role for Notch in Wnt/ β -catenin signalling. The membrane bound-Notch was shown to directly associate with unphosphorylated β -catenin that preceded subsequent degradation in lysosomes (Kwon et al., 2011). Expression of Notch1 in *Apc*-driven mouse models revealed an unexpected conversion of high-grade adenoma to low-grade adenoma via Wnt signalling suppression (Kim et al., 2012a). Together, the data suggest a complex interplay between Notch and Wnt/ β -catenin signalling in intestinal tumorigenesis.

mTOR. The serine/threonine kinase mTOR is a key component of the mechanistic target of rapamycin complex 1 (mTORC1) that is implicated in cell growth control by regulating protein synthesis. Previous studies have shown that *Apc* mutated intestinal tumours are dependent on mTORC1 signalling required for tumour growth (Faller et al., 2014; Fujishita et al., 2008). Faller and colleagues showed that mTORC1 is required for the proliferation of *Apc*-deficient cells by mediating translational elongation through eEF2 kinase (Faller et al., 2014). Moreover, reduced mTORC1 signalling in Paneth cells has been shown to enhance ISC function in response to caloric restriction (Yilmaz et al., 2012). In addition, autophagy, a process regulated by mTORC1, is believed to negatively regulate Wnt signalling by promoting DVL degradation (Gao et al., 2010). Consistently, a reverse correlation between DVL expression and autophagy is observed in late stages of CRC (Gao et al., 2010). In addition, autophagy has been shown to be an alternative mechanism for β -catenin degradation under stress conditions via the formation of a β -catenin/LC3 complex (Petherick et al., 2013). Taken together, mTOR signalling is believed to be closely associated with Wnt/ β -catenin signalling, which may represent an attractive therapeutic target for Wnt activated CRCs.

1.7 Hypothesis

The current evidence strongly suggests that CRC initiation and progression is closely linked to alterations in the ISC pool (Barker et al., 2009; Merlos-Suarez et al., 2011). Therefore, unravelling novel genes that contribute to ISC regulation is important for better understanding of both intestinal homeostasis and CRC biology. Wnt/ β -catenin signalling is the gatekeeper for ISC regulation. Precise control of the

Wnt pathway is critical for ISC maintenance and differentiation. In addition to the central key players of the Wnt pathway components, several new regulators such as *RSPO/LGR* and *RNF43/ZNRF3* have been recently identified as additional regulatory mechanisms required for Wnt signalling. Interestingly, some of the Wnt negative regulators, such as *RNF43/ZNRF3* and *AXIN2*, are direct Wnt transcriptional targets that are expressed at the ISC crypt compartment. This highlights an important negative feedback mechanism of the Wnt pathway for ISC regulation.

As discussed earlier, aberrant Wnt activation is observed in the majority of human CRCs. In most MSS tumours, Wnt/ β -catenin signalling activation is driven by mutation in the *APC* gene that result in hyperproliferation of the stem cell compartment (Figure 1.6). In the subset of non-*APC* mutated CRCs several other genetic alterations in Wnt antagonists have been described, such as *AXIN2* and *RNF43* (TCGA, 2012). This data suggests that, the Wnt negative feedbacks, generated by genes expressed at the stem cell compartment, are fundamental to maintain intestinal homeostasis.

To gain insight into the Wnt negative feedback mechanism in CRCs, I analysed the publicly available genome-wide transcriptomic data from a large cohort of human CRCs (TCGA, 2012). Interestingly, hierarchical clustering analysis revealed a cluster of genes that are enriched in previously reported ISC markers, Wnt targets and/or Wnt inhibitors (see Chapter 3). From here onwards, this cluster will be referred to as the “Stem-Cell/Wnt cluster”. Notably, differential expression of various genes is observed among normal, MSI and MSS CRCs samples. In particular, most genes in this cluster show downregulated expression in MSI CRCs compared with MSS samples. This supports the notion that inhibition of the Wnt negative feedback mechanism is the alternative Wnt activating mechanism in MSI CRCs. We hypothesise that some of the genes within this “Stem-Cell/Wnt cluster” are Wnt inhibitors responsible for a negative feedback loop to fine-tune the levels of Wnt/ β -catenin signalling for tissue homeostasis (Figure 1.6).

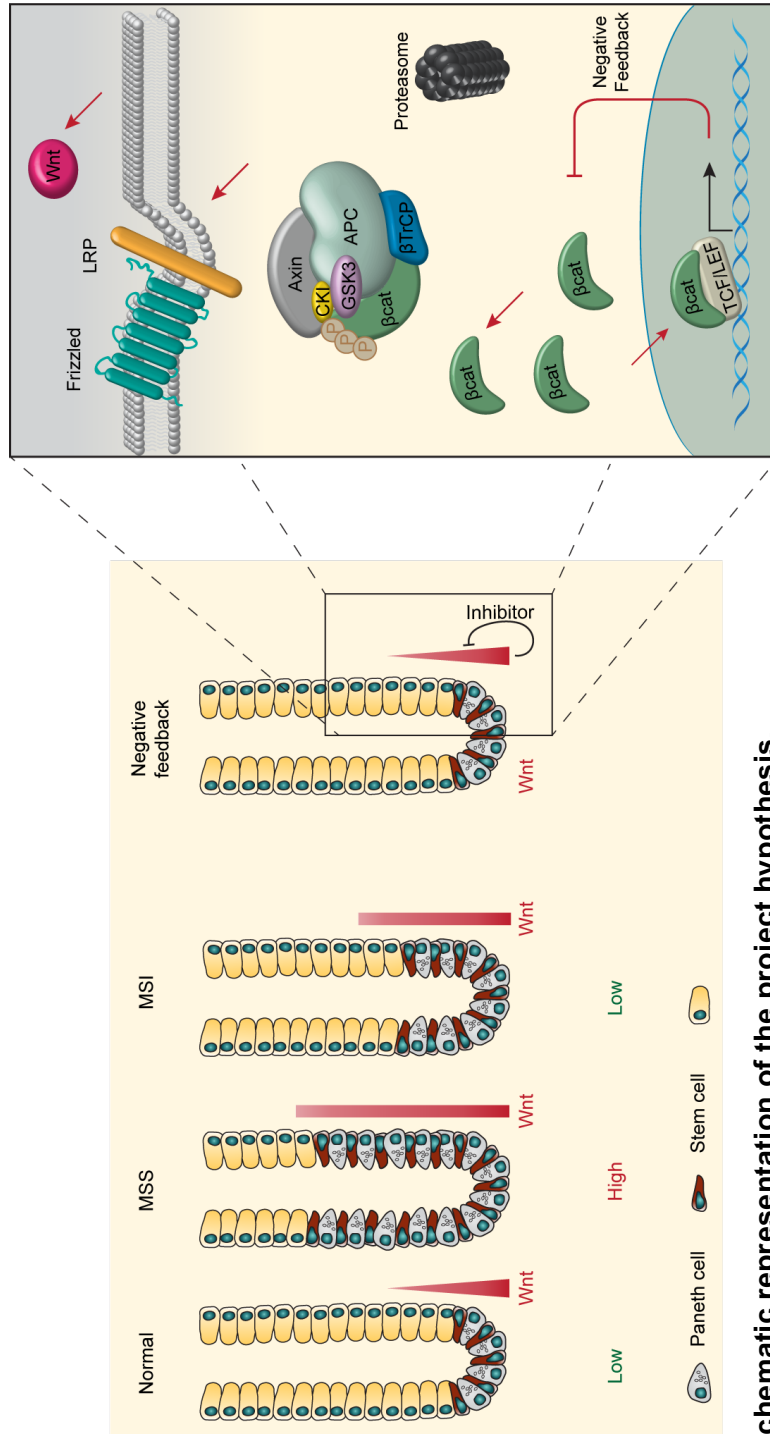


Figure 1.6 Schematic representation of the project hypothesis.

Wnt signalling expression is restricted to the bottom of the crypt in healthy tissue. The majority of MSS CRC harbours APC mutations, resulting in hyperactivation of Wnt signalling and crypt stem cell expansion. MSI CRCs, on the other hand, activate Wnt signalling via alternative mechanism by targeting Wnt agonists or antagonists, and appear to have lower level of Wnt activation compared to MSS tumours. Several Wnt inhibitors have been reported as direct Wnt transcriptional targets, suggesting that these Wnt target inhibitor create a negative feedback mechanism to fine-tune Wnt signalling for normal stem cell homeostasis (inset). Wnt signalling is regulated at different levels by multiple inhibitors. These inhibitors act either intracellularly to modulate components of the signal transduction machinery or extracellularly to modulate ligand-receptor interactions. Red arrows indicate various potential levels of the signalling cascade at which the inhibitors can act on.

1.8 Aim

The aim of my PhD thesis is to identify and characterise novel Wnt regulator(s) from the “Stem-Cell/Wnt cluster” that may play a crucial role in both ISC homeostasis and CRC development. I aim to gain insights into CRC biology through identification and characterisation of novel, potential Wnt inhibitors.

I have shortlisted two potential novel Wnt regulators, ZAK and SH3BP4 based on the comprehensive analysis described in Chapter 3. The specific aims of my project are to determine: 1) the regulatory role of these two genes in the Wnt signalling pathway, and 2) their functional significance in intestinal homeostasis, regeneration and tumourigenesis.

Chapter 2. Materials & Methods

2.1 Cell culture, transfection and TOPFlash assay

HEK293T, LS174T and SW480 cells were maintained in DMEM GlutaMAX (Gibco) supplemented with 5% foetal bovine serum (FBS) (Gibco) and 100 units/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). All cell lines were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Cells were seeded in plates 24hrs before transfection and plasmids were transfected using Polyethylenimine (Polysciences) according to the manufacturer's instructions. For the TOPFlash luciferase assay, cells were seeded at a density of 1x10⁵ cells/well in a 24-well plate one day before transfection. The cells were then transfected with 200ng of TopFlash or FopFlash plasmid constructs (optimal and mutate TCF luciferase-reporter constructs, respectively). The former consists of 5 binding motifs for TCF, cloned upstream of a minimal promoter, and the latter harbours mutations in those motifs as previously described (Korinek et al., 1997). Transfection efficiency was normalised against the co-transfected renilla luciferase activity (10ng/well). Control or Wnt3A-conditioned medium was added to the cells 24hrs post-transfection, as indicated. Treated cells were lysed after 16hrs using luciferase lysis buffer (Promega), and luciferase activity was measured using the Dual-Luciferase-reporter assay system (Promega) in a luminometer. MAPK inhibitors (PD0325901, XMD8-92, SCH772984, SP600125 – Selleckchem) and Rapamycin (Sigma) or vehicle dimethyl sulfoxide (DMSO) were added simultaneously with Wnt3A as indicated.

2.2 Cells gene Editing by CRISPR/Cas9 System

To generate ZAK and SH3BP4 knock-out HEK293T cells, single guide RNAs (sgRNA) were designed for specific target regions, as previously described (Ran et al., 2013). HEK293T cells were transfected with plasmids encoding Cas9 and sgRNAs (PX459, #62988, Addgene, a gift from Feng Zhang lab). ZAK was targeted using the gRNA: 5'-TCGAGCCAAATGGATATCAC-3' and SH3BP4 with the gRNA: 5'-GGGCGACCATCTCTACGTCT-3'. LS174T cells were targeted for SH3BP4 deletion with the same sgRNA. 48hrs after transfection, cells were selected using 2µg/ml puromycin. Single, puromycin-resistant cells were selected and expanded for

genomic DNA extraction. The targeted locus was then polymerase chain reaction (PCR) amplified and subcloned into a TA-cloning vector. Indel mutations were confirmed by sequencing, and loss of protein by Western blot (see primers for genotyping in Table 1 and antibodies used in Table 2).

Primer	Forward	Reverse
CRISPR/Cas9 genomic engineering		
SH3BP4 sgRNA	AGACGTAGAGATGGTCGCCCCGGTGTTCGT CCTTTCCACAAGAT	—
ZAK sgRNA	GTGATATCCATTTGGCTCGACGGTGTTCGTC CTTTCCACAAGAT	—
T7_Cas9	TAATACGACTCACTATAGGGAGAATGGACTAT AAGGACCACGAC	GCGAGCTCTAGGAATTCCTAC
T7_Sh3bp4::FLAG_sgRNA	TTAATACGACTCACTATAGGGGGACTCTGCC CACCAGACC	—
Sh3bp4::FLAG_sgRNA	CACCGCCCACTCCAGTGAGCAGTA	AAACTACTGCTCACTGGAGTTGGGC
Sequencing_Sh3bp4::FLAG	TCGTGCGGCAGCGTCAGATGTGTATAAGAGAC AGAGATTGGGGACAGCTACCGA	GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGGGAGAGATGATGGGCCAAGT
Sequencing_Zak	TCGTGCGGCAGCGTCAGATGTGTATAAGAGAC AGTTTCTGAACATCATCGGCCT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGTTGGGAAGGAGCCTCATGGA
Cloning		
SH3BP4::FLAG	GTGGCGGCCGCTCGAGGCCATGGCGGCTCA GCGGATCCGA	TCCTTGTAGTCTCGAGCAATCACGAAGTCGT CCTGGTCC
MACC1::FLAG	GTGGCGGCCGCTCGAGGCCATGCTAATCACT GAAAGAAAAC	TCCTTGTAGTCTCGAGCTACTTCTCAGAAAT GGAGAAT
ZAK::FLAG	GGACTCAGATCTCGAGATGGATTACAAGGAT GACGACGATAAGTCGTCTCTCGGTGCCTCCT TTGTG	GAAGCTTGAGCTCGAGTCAAAAGTTTCTCCAT CCACG
SH3BP4^{ADD}	GTGGCGGCCGCTCGAGGCCATGGCGGCTCA GCGGATCCGA	TCCTTGTAGTCTCGAGCCTCCAGCAGCACCG AGGTGCTCA
SH3BP4^{ΔN}	GTGGCGGCCGCTCGAGGCCATGCCCTTGAA CTACCGGAACTCAACAC	TCCTTGTAGTCTCGAGCAATCACGAAGTCGT CCTGGTCC
SH3BP4^{W92A}	CACATCTGGCGGTGAGCGCTGGTACGCACA CAAC	GTTGTGTGCGTACCACGCCTCACGCCAGAT GTG
SH3BP4^{ΔSH3}	GTGTCCAGCCTCAAGCTGGTGGTCGGCAGG GCCCCGG	CCTGCCGACCACAGTTGAGGCTGACACC GGGCGG
SH3BP4^{ΔZU5}	CAGACCCAAGCCGTGCTTTTAGCAAAAGCA CAGTG	GCTTTTGCTAAAAAGCACGGCTTGGGTCTGG CCCCA
ZAK^{K45A}	GAGGTGGCTGTAGCGAAGCTCCTCA	—
Genotyping		
Sh3bp4:FLAG KI	TGACTGAGGGACCTCACC	GGATCCCACTCACTTGTCTATC
Sh3bp4^{fl/fl} WT	GTCAAGGTGTAGGAGAGGGAGG	GGCAAAGGACTCAGAGGAATG
ZAK WT	CAAATGGATATCACAGGACA	TTGGAAGGAGCCTCATGGA
ZAK M1	AATGGACAAGGAGGTGGCTG	TTGGAAGGAGCCTCATGGA
ZAK M2	TCGAGCCAAATGGATACAC	TTGGAAGGAGCCTCATGGA
RT-qPCR		
Sh3bp4 mouse	GTGGCGTCCGTCTAGAAAA	GACTCGTAGGCGTCCATCTG
Zak mouse	GAGTGGAGGTGGGTAGCTCCGGCA	CCCAAGCTGTGCTTAGTCATC
Lgr5 mouse	CAAGCCCTGTGAGCACCTAT	ACTGGAGACCCCCATGAGAA
Actin mouse	ATGCTCCCCGGGCTGTAT	CATAGGAGTCTTCTGACCCATTC
AXIN2	AGTGTGAGGTCCACGAAAC	CTTCACACTGCGATGCATTT
CCND1	CTGGCCATGAACCTACCTGGA	CTCCGCCTCTGGCATTCTTGG
MYC	TCTCCTTGACAGCTGCTTAG	GTCGTATCGAGGTCATAG
ACTIN	GAGCGCGGCTACAGCTT	TCCTTAATGTACGCACGATTT

Table 1. List of primers used.

Antibodies	Host species	Dilution	Source
β -Catenin (Active)	Mouse	1:1000	Millipore (05-665)
β -Catenin (Total)	Rabbit	1:1000	Santa Cruz (sc-7199)
Alexa - Fluor 488	Mouse	1:1000	Invitrogen (A11029)
Alexa - Fluor 568	Rabbit	1:1000	Invitrogen (A11036)
APC	Mouse	1:1000	Calbiochem (OP44)
Cleaved Caspase 3	Rabbit	1:200	Cell Signalling (9664)
CYCLIND1	Rabbit	1:75	Cell Signalling (2978)
FLAG	Mouse	1:1000	Sigma (A2220)
GAPDH	Rabbit	1:1000	Santa Cruz (sc-25778)
Ki67	Rabbit	1:350	Abcam (ab16667)
LAMINA	Rabbit	1:250	Abcam (ab11575)
LYSOZYME	Rabbit	1:2000	DAKO (a0099)
Phospho-p70S6	Rabbit	1:1000	Cell Signalling (9205)
Mouse IgG- HRP	-	1:5000	GE (NXA931)
Rabbit IgG - HRP	-	1:5000	GE (NA934)
SH3BP4 (Human)	Mouse	1:500	Santa Cruz (sc-393730)
SOX9	Rabbit	1:500	Millipore (AB5535)
TCF4	Goat	1:1000	Santa Cruz (sc-8631)
Total p70S6	Rabbit	1:1000	Cell signalling (9202)
ZAK (Human)	Rabbit	1:1000	Bethyl (A310-705A)
ZAK (Mouse)	Mouse	1:1000	Abcam (ab57318)

Table 2. List of antibodies

2.3 DNA cloning and plasmids

Full-length *ZAK*, *SH3BP4* and *MACC1* were amplified by PCR from HEK293T cell genomic DNA. Briefly, 50ng of DNA was amplified using Phusion® High-Fidelity PCR Master Mix (Biolabs) using the following reagents: 0.5 μ M forward and reverse primers in 3% DMSO in volumes of 25 μ l. The thermocycler consisted of 35 cycles of 98°C for 30s, 56-62°C (depending on the construct) for 30s, and 72°C for 30s per kilobase of amplification. PCR products were visualised and size verified on a 1%

w/v agarose/TAE (40mM Tris-acetate, 20mM acetic acid, 1mM EDTA (ethylenediaminetetraacetic acid) in deionized water) gel containing 5ng/mL ethidium bromide. PCR products were cloned into pcDNA-*FLAG* plasmids using the In-Fusion® DH Cloning Kit, according to the manufacturer's instructions. Cloning primers with a XhoI cutting site were used to confirm insertion.

The SH3BP4 dead domain constructs were generated using the In-Fusion® DH Cloning Kit with primers specifically designed for each domain. Each primer contained a homology arm of 15 base pairs (bp) (see primers in Table 1).

The constructs with site directed mutagenesis were generated by PCR of the original construct with mutagenic primers (Table 1). Phusion® High-Fidelity PCR Master Mix was used and non-mutated parental DNA template was digested with the restriction endonuclease DpnI.

2.4 Immunoblotting and immunoprecipitation

Cells were lysed in cold lysis buffer containing 150 mM NaCl, 30 mM Tris (pH 7.5), 1 mM EDTA, 1% Triton X-100, 10% Glycerol, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 0.5 mM DTT (dithiothreitol), protease inhibitor cocktail tablets (EDTA-free) (Roche), and phosphatase inhibitor cocktail tablets (Roche). Lysates were pelleted for 30 min at 13200 r.p.m. and supernatants kept for protein quantification (Bradford assay). Equal amounts of cellular protein were resolved in 10% sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked using 5% milk (OXOID) or 5% bovine serum (BSA) (Sigma) for phosphorylated proteins immunoblots, in Tris-buffered saline TBS (50mM Tris, 150mM NaCl, pH7.6) containing 0.1% Tween-20 (Sigma) (TBST) for 1 hour, and primary antibodies were added in blocking solution. See full list of antibodies and dilutions in Table 2. Primary antibody incubations were carried out at 4°C overnight. After washing with TBST, the appropriate HRP-conjugated secondary was added (1:5000 in blocking buffer) for 2 hours at room temperature. Antibody binding was detected using chemiluminescence ECL Prime Western Blotting Substrate (GE Healthcare).

For immunoprecipitation experiments, cells were washed and collected with the above indicated lysis buffer. After clarification by centrifugation (14,000 rpm for 30 min at 4° C), cellular lysates were precleared with IgG-agarose beads (Millipore) for at least 2h at 4°C. Immunoprecipitation was performed by incubating the cellular lysates with the desired antibodies at 4°C overnight. SH3BP4 Knock-out cells (Δ SH3BP4) were generated by CRISPR to use as negative control and to validate the specificity of the interaction. Earlier truncation and loss of protein were confirmed by sequencing and western blot, respectively. Protein G PLUS-Agarose beads (Santa Cruz Biotechnology) were added and incubated at 4°C for 2-4hrs. Immunocomplexes were washed with cold lysis buffer six times, and resuspended in Laemmli-SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β -mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue). cocktail tablets (EDTA-free) (Roche), and phosphatase inhibitor cocktail tablets (Roche).

2.5 Crypts/Villi fractionation

Small intestine was washed with cold PBS and cut into small pieces. Sequential incubations with 1mM EDTA for 20min at 4°C were performed. The resulting fractions of crypts and villi (in increasing purities) were passed through a 70 μ m cell strainer each time. Fractions from above and below the strainer were collected and checked under the microscope for purity. Fractions of similar purity were combined, and RNA extraction was performed using the RNeasy® Kit-Qiagen, according to the manufacturer's protocol.

2.6 Real-time quantitative RT-PCR

1 μ g of RNA was reverse transcribed to cDNA using the cDNA synthesis-Thermo scientific kit, following the manufacturer's instructions. qPCR was performed in triplicates in a 15 μ l reaction mixture containing 7.5 μ l of 2X SYBR green, 8 μ M of each primer and 25ng of cDNA. The reaction mixture without template cDNA was run as a control. After 40 cycles of cDNA amplification, samples were normalized to β -actin and data were expressed as mean \pm standard error.

2.7 Immunofluorescence

Cells were grown on poly-L-lysine-coated (Sigma) glass coverslips in 12-well, fixed with 4% paraformaldehyde (PFA) for 15 min, and permeabilised using 0.5% Triton X-100 in PBS for 10 min. Cells were blocked with 1% BSA in PBS for 1h before overnight incubation with primary antibodies at 4°C. Cells were washed three times with PBS and incubated with secondary antibodies conjugated to Alexa-Fluor 488 or 568 at room temperature for 1h in the dark. Cells were washed three times with PBS and stained with DAPI for 10 min. Coverslips were washed another three times with PBS and were then mounted with Aqua Poly/Mount (Polysciences). Images were taken using a Leica SPE confocal microscope. Each fluorophore was imaged separately using 405, 488 and 561 channels. Confocal images were taken as Z-stacks and processed using Fiji (Schindelin et al., 2012).

2.8 Immunohistochemistry

Small intestine and colon tissues were fixed in 10% buffered formaldehyde for 16hrs time and embedded in paraffin. For staining, 4µm sections were de-paraffinized using xylene and rehydrated through a graded series of ethanol. Antigen retrieval was performed for 20 min at high temperature in either 0.01M citrate buffer (pH6) or Tris-EDTA (10mM Tris base, 1mM EDTA solution, pH9). Samples were blocked using 1% BSA and incubated overnight with the desired antibody or negative control at 4°C. Finally, slides were incubated with the secondary antibody for 1h and washed three times with PBS. For colorimetric staining with diaminobenzidine (DAB) slides were incubated with peroxidase substrate, and mounted. Protein expression was visualised using a bright-field microscope.

2.9 RNA *in situ* hybridization

In situ hybridisation (ISH) for *Lgr5*, *Olfm4*, *Axin2*, *Sh3bp4*, *Zak* was performed using the RNAscope FFPE assay kit (Advanced Cell Diagnostics, Inc., Hayward, CA, USA) according to the manufacturer's instructions. Briefly, 4µm formalin-fixed, paraffin-embedded tissue sections were pre-treated with heat and protease digestion before hybridisation with a target probe. Thereafter, an HRP-based signal amplification system was hybridised to the target probe before colour development

with 3,3'-diaminobenzidine tetrahydrochloride (DAB). The housekeeping gene *Ppib* served as a positive control. The *DapB* gene, which is derived from a bacterial gene sequence, was used as a negative control.

2.10 Intestinal organoid culture and imaging

Organoids were established from freshly isolated murine small intestine. Intestines were opened longitudinally, washed with PBS and incubated in cold PBS containing 2mM EDTA for 30 minutes under agitation. The mixture of crypts and villi were passed through 70µm cell strainer multiple times until a pure fraction of crypts was obtained. Crypts were counted using a microscope and 200 crypts were seeded in 20 µl of Cultrex® BME Type 2 RGF PathClear (Amsbio, 3533-010-02) in individual wells of a 24-well plate and cultured as previously described (Sato et al., 2009). The organoid basal media contains EGF (Invitrogen PMG8043), Noggin, R-spondin and Wnt3A. Noggin and R-spondin conditioned media were generated from HEK293T cells. Wnt3A conditioned media was generated from L cells. Images were acquired using EVOS FL Cell Imaging System (Life Technologies) and processed using Fiji (Schindelin et al., 2012).

2.11 Mouse experiments

All animals were maintained with appropriate care according to the United Kingdom Animal Scientific Procedures Act 1986 and the ethics guidelines of the Francis Crick Institute.

The list of transgenic mice used is shown in Table 3.

Mouse strains	Genetic Background	Reference
<i>Tg(CAG-Flpo)1Afst</i>	C57BL/6	(Kranz et al., 2010)
<i>Apc^{min}</i>	C57BL/6	(Moser et al., 1990)
<i>Villin^{CreERT2}</i>	C57BL/6	(el Marjou et al., 2004)
<i>Sh3bp4^{tm1a(EUCOMM)Wtsi}</i>	C57BL/6	Wellcome Trust Sanger Institute, 2010
<i>Sh3bp4^{fl/fl}</i>	C57BL/6	In this study
<i>Sh3bp4^{fl/fl} Apc^{min}</i>	C57BL/6	In this study
<i>Sh3bp4^{fl/fl} Villin^{CreERT2}</i>	C57BL/6	In this study
<i>Zak^{-/-}</i>	(CBAxC57BL/6)F1	In this study

Table 3. List of transgenic mice used

2.11.1 Mouse generation by CRISPR/CAS9-mediated genome engineering

ZAK-null (*ZAK*^{-/-}) mouse. Generation of *Cas9* mRNA and sgRNA were performed as previously described (Yang et al., 2014). *In vitro* transcription of *Cas9* mRNA was done using the mMESSAGE Machine T7 Kit and MEGAclear kit following the manufacturer's instruction. sgRNA was transcribed using the MEGAshortscript T7 kit, as per the kit protocol. C57BL/6 X CBA(F1) female mice were used as embryo donors and foster mothers. Superovulated female mice (7–8 weeks old) were mated to males, and fertilised embryos were collected from oviducts. *Cas9* mRNAs (10ng from a 100 ng/μl stock) were injected into zygotes, and sgRNA (25ng from 50 ng/μl stock) was injected into the cytoplasm of fertilised eggs with well recognised pronuclei in M2 medium (Sigma). Zygote injections were performed by the Genetic Manipulation Service at the Francis Crick Institute. Genotyping was performed by targeted gene sequencing in the Illumina Miseq System according to the manufacturer's instructions (see primers in Table 1). Briefly, PCR products were purified using Ampure XP reagent (A63881, Beckmann-Coulter) at 1.8X sample concentration. Sequencing libraries were created by ligating adaptor sequences to the pooled DNA using the Ion Plus Fragment Library Kit (4471252, Life Technologies). 50 ng of purified PCR product was end-repaired and amplicons with adaptors were purified with Ampure XP reagent at 1.2X sample concentration. FastQ reads were mapped to the *Mus musculus* genome version 9 (mm9).

***Sh3bp4::FLAG* knock in (KI) mouse.** *In vitro* transcription of *Cas9* mRNA and sgRNA were performed as described above. A single oligo was synthesised by Sigma. Zygotes of C57BL/6 mouse background were injected with a mixture composed of 10ng of *Cas* mRNA, 25ng of sgRNA and 20ng of the donor oligo (200 ng/μl). Genomic DNA from the resulting animals was genotyped as described above.

2.11.2 Genotyping and PCR amplification

Genotyping was performed by PCR amplification of genomic DNA extracted from ear punch biopsies taken from mice aged three weeks. Appropriate primers were used for amplification of each allele (Table 1). Ear punch biopsies were

digested in 200µl of lysis buffer (10mM Tris 7.5, 100mM NaCl, 10mM EDTA, 0.5% Sarkosyl) at 55°C overnight. 200ng of DNA was then amplified using MyTaq™ Red Mix (BIOLINE) 0.4µM forward and reverse primers in reaction volumes of 25 µl. The thermocycler consisted of 35 cycles of 95°C for 30s, 56-62°C (depending on the primer) for 30s, and 72°C for 1 minute per kilobase of amplification. PCR products were then visualised and size verified on a 2% w/v agarose/TAE gel with 5ng/mL ethidium bromide.

2.11.3 EdU injections

5-ethynyl-2'deoxyuridine (EdU) (Life Technologies) was injected intraperitoneally (0.3mg/10g of mouse weight) from a 10mg/ml stock solution. Mice were culled by schedule 1 procedure (SK1) at the desired time point.

2.11.4 Induction of Cre

Tamoxifen (Sigma) was injected intraperitoneally (1.5mg/10g mouse weight) from a 20mg/ml stock solution, over 3 consecutive days. Mice were culled at the desired time point by SK1.

2.11.5 Radiation injury model

Mice were exposed to controlled 10 Gray (10Gy) total body ionising radiation to induce damage. Mice were culled at the desired time points by SK1.

2.12 Statistical analysis

All data are presented as the mean \pm the standard error of the mean. N refers to independent experiments. TOPFlash luciferase assay and qPCR measurements were performed in three technical replicates in at least three independent experiments. *In vivo* data corresponds to at least three independent experiments, if not otherwise stated. Radiation experiments and administration of substances were performed in groups of at least two animals (one wild-type control mouse and one

full knock-out or conditional knock-out mouse, as indicated) at least in three independent experiments. Histological analysis and cell counting were performed blind.

Quantification of positive cells for the indicated staining in villi and crypts were performed in 30 villi or crypts per animal in at least 3 animals per group, unless indicated otherwise. Cells were counted in the jejunum, if not indicated otherwise. The number of positive cells for the indicated staining in adenomas were performed in tissue sections. 5 adenomas, with sizes between 0.8 to 1 mm and similar grade of dysplasia were analysed from 6 different animals per group. Measurement of villi and crypts length were performed in 30 villi/region in 3 animals per group.

Statistical analyses were performed using Graph Pad Prism software. Normal distribution of data was determined using the D'Agostino and Pearson omnibus test. For parametric data, statistical significance was determined using a student's unpaired, two-tailed t-test. In cases where more than two groups were being compared, then a one-way ANOVA was used. In instances where the N was too small to determine normal distribution or the data were non-parametric, a two-tailed Mann-Whitney U-test was used. P values of less than or equal to 0.05 were considered statistically significant and are represented as *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, non-significant (ns- $p > 0.05$).

Chapter 3. Screening for novel Wnt regulators in colorectal cancer

3.1 Introduction

In the adult intestine, it is well established that Wnt signalling plays complementary roles in physiology and pathology. Wnt signalling maintains the crypt stem cell compartment by controlling the level of the key downstream modulator β -catenin for signal transduction through the cytoplasmic β -catenin destruction complex. However, mutations in key components of the Wnt pathway results in constitutive Wnt activation, which is a frequent event in CRC. As discussed in the introduction chapter, human CRC can be broadly classified into four molecular subtypes (CMS1-4) based on their gene expression profiles (Guinney et al., 2015). The CRCs cohort from the TCGA network has been broadly divided into two molecular subtypes, microsatellite instability (MSI) and microsatellite stable (MSS), based on their hypermutation profiles (TCGA, 2012). The vast majority of CRCs are MSS, where loss of the tumour suppressor APC signals the initiation of adenoma formation (Nagase and Nakamura, 1993), as has been suggested for the molecular subtypes CMS2,3 and 4. On the other hand, around 10-15% of CRCs are caused by defective DNA mismatch repair machinery and these are the MSI group (CMS1). These are characterised by the presence of insertions or deletions of nucleotides in microsatellite repeat regions, which are widespread across the genome (Ionov et al., 1993; Thibodeau et al., 1993).

Wnt signalling is aberrantly upregulated in both MSS and MSI tumours (TCGA, 2012). The aberrant Wnt signal activation in MSS CRC is well characterised, with evidence of stem cell-specific loss of APC as the origin of intestinal neoplasia (Barker et al., 2009). Accumulation of other alterations such as additional mutations, epigenetic silencing, microenvironmental signals, or pathway crosstalk are further required to confer tumourigenesis and the progression from adenoma to carcinoma (Fearon, 2011; Zeuner et al., 2014). In MSI-CRCs, mutations in key components of the Wnt pathway, such as *APC*, *β -catenin* and *Axin2* are only observed in a

subpopulation (TCGA, 2012), suggesting an alternative Wnt-activating mechanism in this CRC subtype.

Negative feedback control mechanisms are crucial in regulating various biological signalling pathways (Freeman, 2000). As discussed in chapter 1, precise control of Wnt signalling in the intestinal stem cell compartment is governed by a number of Wnt inhibitors and negative feedback mechanisms at different subcellular levels of the cascade. For example, several extracellular proteins such as SFRPs and DKKs, antagonise Wnt signalling by inhibiting Wnt protein engagement to Fz or LRP receptors (Cruciat and Niehrs, 2013). The secreted protein NOTUM also antagonizes WNT signalling by deacylating Wnt proteins (Kakugawa et al., 2015). In addition, the stem cell E3 ligases, ZNRF3 and RNF43, inhibit Wnt signalling by inducing rapid endocytosis of WNT receptors (Hao et al., 2012; Koo et al., 2012). The latter has also been shown to regulate WNT via sequestering the transcription factor TCF4 to the nuclear membrane (Loregger et al., 2015). The negative Wnt regulator AXIN2 represents another classic, negative feedback regulation that is upregulated upon Wnt activation to control the signalling activity (Lustig et al., 2002).

Loss of the negative feedback loop could result in hyperactivation of WNT signalling and initiate tumour growth. In fact, mutations in several WNT inhibitors have been reported in some CRCs, as discussed in chapter 1. For example, somatic mutations in *RNF43/ZNRF3* are found in some human CRC cell lines and patients (Giannakis et al., 2014; Koo et al., 2012), as well as in many other human cancer types (Assie et al., 2014; Furukawa et al., 2011; Jian Wu et al., 2011 ; Jiang et al., 2013; Ong et al., 2012; Ryland et al., 2013; Yan et al., 2017). Inactivating mutations of *AXIN2* are found predominantly in hypermutated CRCs (Koinuma et al., 2006). Interestingly, both *RNF43* and *AXIN2* Wnt inhibitors are expressed in the Wnt-active intestinal crypt base (Koinuma et al., 2006; Koo et al., 2012), suggesting the importance of the negative feedback loop for Wnt signalling control during intestinal stem cell homeostasis. Based on the close link between stem cell and cancer biology in the context of Wnt signalling, I aim to identify and characterise novel Wnt inhibitors that are involved in both intestinal stem cell control and tumourigenesis.

Recent advances in Whole Genome Sequencing have generated large amounts of sequencing data from many series of human tumours. The studies generated by the Cancer Genome Atlas (TCGA) and other consortia have provided valuable resources for cancer research. Such findings have uncovered novel

mutations in a large set of genes, many of which have not been characterised. The next step is to systematically analyse the data for a better understanding of human cancer. In 2012, the TCGA published a genome-scale analysis of a large cohort of human CRC that allows us to obtain the global gene expression profile of the cancer samples (TCGA, 2012). Hierarchical clustering analysis of the expression data revealed a differentially expressed gene cluster that consists of many reported intestinal stem cell markers, Wnt targets and Wnt inhibitors. From now on, this will be referred to as the “Stem-Cell/Wnt cluster”. I hypothesise that a subset of negative Wnt negative regulators expressed by stem cells are present within this cluster, and that deregulation of these inhibitors will interrupt the negative feedback loop and cause aberrant Wnt activation and cancer. Comprehensive analysis of the Stem-Cell/Wnt cluster identified two novel putative Wnt inhibitors: the mixed-lineage zipper sterile- α -motif kinase (ZAK) and the SH3 binding domain protein 4 (SH3BP4).

3.2 Results

3.2.1 TCGA hierarchical clustering reveals a “Stem-cell/Wnt cluster”

The TCGA had previously conducted genome-scale analysis of 276 CRC samples by analysing exome sequence, DNA copy number, promoter methylation, mRNA and microRNA expression (TCGA, 2012). All data was deposited at the Data Coordinating Center for public access (<http://cancergenome.nih.gov/>). To study the differential expression within a large cohort of CRC samples, we obtained the gene expression data of 173 colon samples from TCGA, all with known *MLH1* silencing status and defined mutation profiles. The data were mean centred and genes with 2-fold variation in at least 10 arrays were selected, resulting in 8379 differentially expressed genes. Hierarchical clustering analyses of these genes readily segregated the samples into different subgroups, including normal (19 cases), MSI (37 cases, based on *MLH1* downregulation and hypermutation profile) and MSS-CRCs (105 cases with non-hypermuted profile and downregulated *APC* expression) (Figure 3.1).

Interestingly, we identified a cluster of 468 genes that showed relatively upregulated expression in MSS-CRCs, compared with both MSI-CRCs and normal tissue. Notably, many of the genes within this cluster have been reported to be

intestinal stem cell markers (e.g. *LGR5*, *ASCL2*, *AXIN2*, *RNF43*) (Barker et al., 2007; Koo et al., 2012; Lim et al., 2013; van der Flier et al., 2009b), Wnt targets (e.g. *LGR5*, *AXIN2*, *ASCL2*, *TCF7*) (Lim et al., 2013; Sato et al., 2009; van der Flier et al., 2009b) and/or Wnt inhibitors (*AXIN2*, *RNF43*, *ZNRF3*, *NKD1*, *NKD2*, *APCDD1*) (Hao et al., 2012; Koo et al., 2012; Lim et al., 2013; Shimomura et al., 2010; Van Raay et al., 2007). We therefore named this cluster “Stem-Cell/Wnt cluster”. We hypothesised that the low expression in normal samples reflects the restricted expression of these genes to stem cell compartment, whilst the high expression in MSS CRCs is a result of a hyperactivated Wnt transcriptional programme. Interestingly, there is an isolated cluster of twelve samples with lower expression of the Wnt signature genes than in MSI and MSS. These samples are metastatic cancers with increased expression of *TGFBI*, a feature of CMS4 subtype. Therefore, I hypothesise that a subset of novel Wnt regulators are present within this Stem-Cell/Wnt cluster, and that these Wnt target genes may function as negative regulators of the pathway for the feedback mechanism

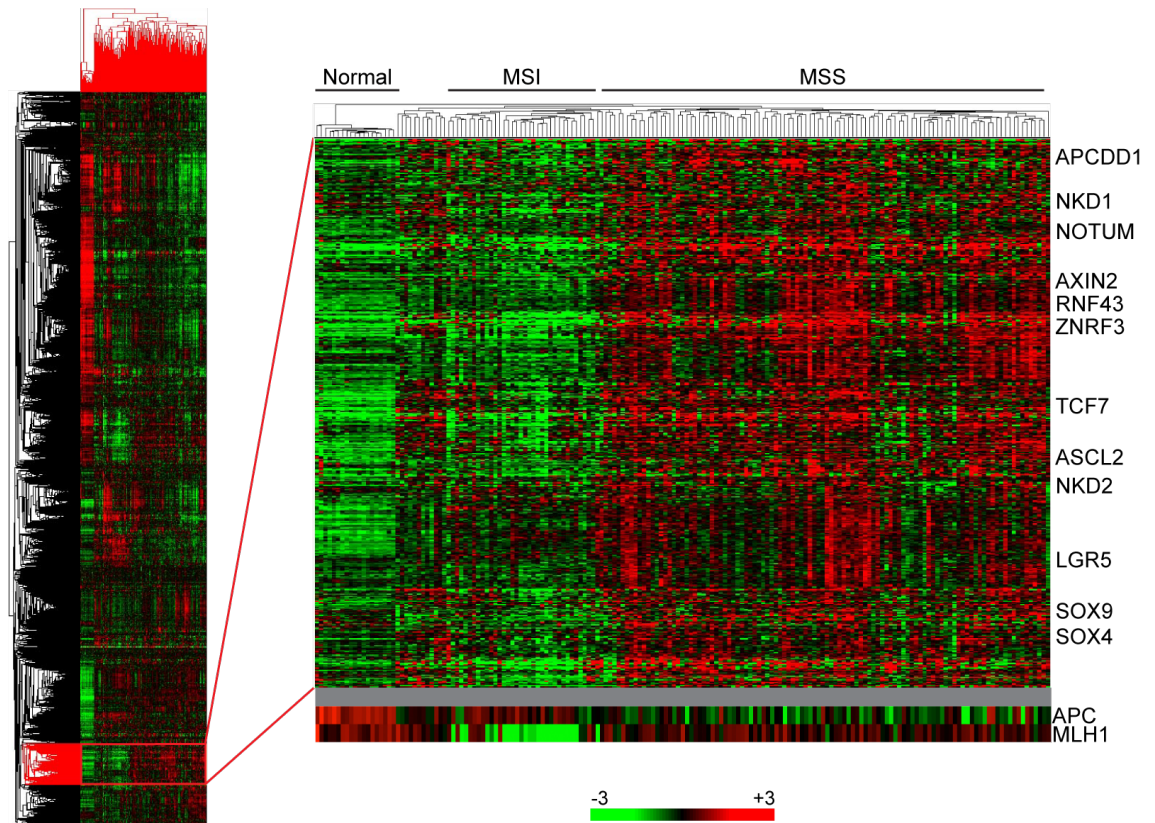


Figure 3.1 TCGA hierarchical clustering reveals “Stem-cell/Wnt cluster”

Heat map showing hierarchical clustering analysis of expression data in 173 CRCs, obtained from TCGA. Three individual subgroups (Normal, MSI- and MSS-CRCs) are clustered together. The Stem-Cell/Wnt cluster is enlarged on the right. Representative stem cell markers, Wnt targets and/or Wnt inhibitors are indicated. Expression profiles of *APC* and *MLH1* are shown at the bottom, which correlates with MSI and MSS status. Scale bar in log₂ ratio is given at the bottom (-3 to +3). Data were displayed using TreeView software. Red, upregulated; green, downregulated.

3.2.2 Identification of novel putative Wnt regulators by comparing the Stem Cell/Wnt cluster with other microarray data

We compared the Stem-Cell/Wnt cluster with other stem cell/Wnt-related microarray data to shortlist putative stem cell-expressed Wnt target genes. Several expression microarray studies characterising the Wnt transcription signature have been published in the past. Specifically, Sansom and colleagues have generated Wnt-activating transcriptomes *in vivo*, using an inducible *Apc* deletion mouse model (*Apc^{fl/fl}*) (Sansom et al., 2004). The expression profile of the intestinal epithelium before and after *Apc* gene deletion was compared to identify hyperactivated Wnt targets. Another study compared the gene expression changes in *ex vivo* intestinal organoid culture upon R-spondin withdrawal (de Lau et al., 2011). As described earlier, R-spondin is a potent Wnt agonist and is an essential component of organoid culture medium to sustain intestinal stem cell maintenance (Sato et al., 2009). R-spondin withdrawal will therefore result in downregulated expression of intestinal stem cell markers and Wnt target genes.

To identify putative Wnt target genes expressed in stem cells in our Stem-Cell/Wnt cluster, data from the two previously published Wnt target transcriptomes mentioned above (*Apc* deletion and R-spondin withdrawal) were retrieved for comparison. It should be noted that the *Apc* deletion *in vivo* experiment was prepared from full thickness intestinal tissues including lamina propria, submucosa and muscle layers, whilst the R-spondin withdrawal experiment was prepared in organoid culture consisting of epithelial cells alone. For the organoid R-spondin withdrawal experiment, the average fold-change in expression of putative Wnt target genes was taken between days 1 and day 3 for comparison. To focus on identifying stem cell/crypt-expressed genes, we first compared our data with the R-spondin array. This identified 51 out of 468 genes with at least 2-fold downregulated expression upon R-spondin withdrawal (Figure 3.2 – right). Among the 51 genes, 26 also showed more than 1.5-fold upregulated expression in the *Apc* deletion microarray study (Figure 3.2 – left and Table 4).

To further shortlist the 26 candidates, we followed a comprehensive analysis represented in Figure 3.3. Firstly, we selected genes that had not been associated with Wnt signalling and/or stem cells and had not been analysed in intestine before.

Secondly, we focused on genes with no more than two potential homologues to avoid redundancy during the mechanistic analysis and *in vivo* functional knock-out studies in mice. Eight genes fulfilled this two criteria: *FAM60A*, *FLJ21865*, *ZAK*, *CYP39A1*, *SH3BP4*, *SPAG4*, *CKAP2*, *GGH*. An extensive literature review finally shortlisted two promising candidates: the mixed-lineage zipper sterile- α -motif kinase (ZAK); and the SH3 binding domain protein 4 (SH3BP4), which will be discussed in detail in chapters 4 and 5 respectively.

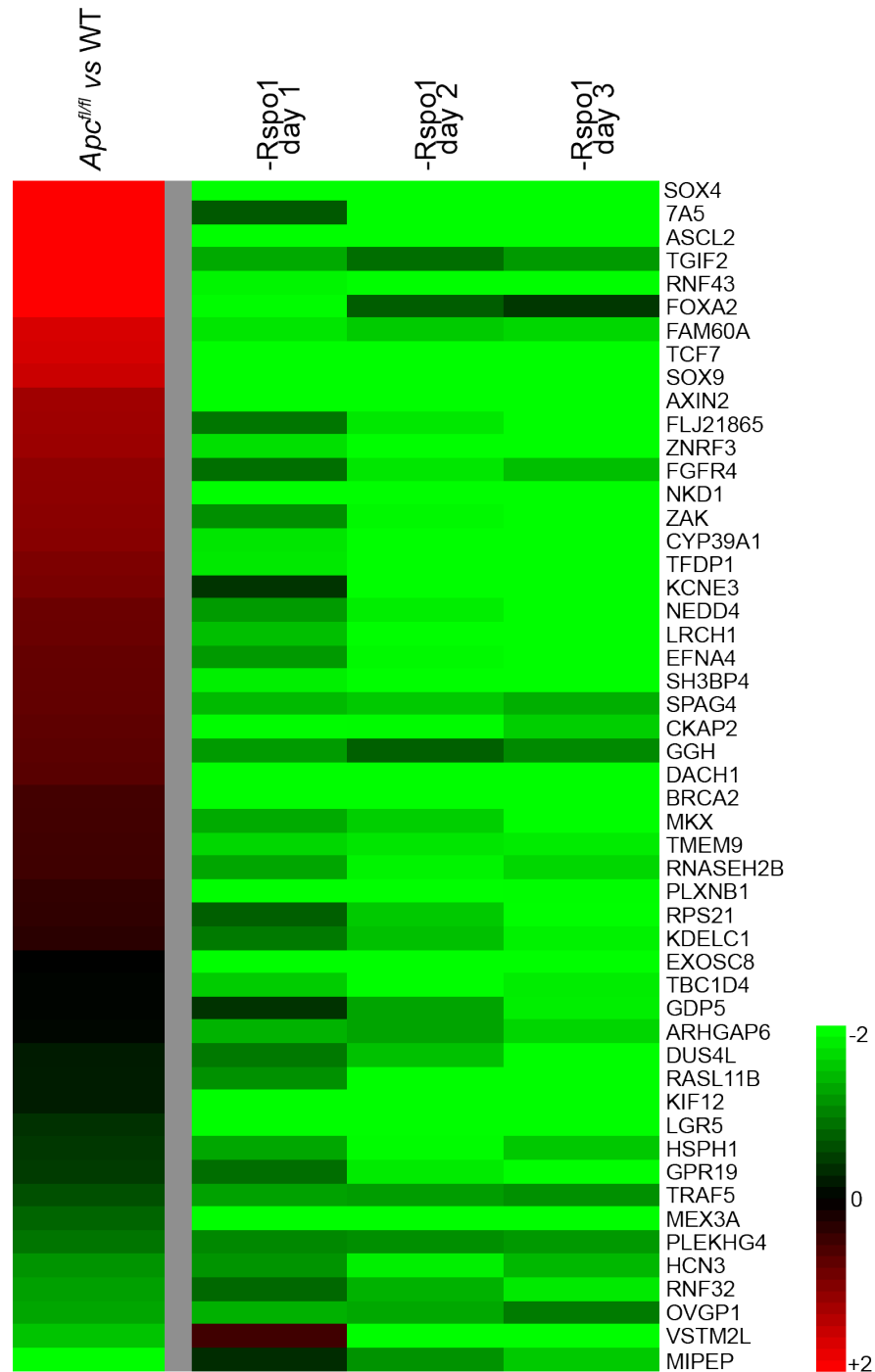


Figure 3.2 Expression pattern of the potential Wnt regulator candidates in stem cell/Wnt-related microarray studies.

Heat map showing 51 potential stem cell/Wnt targets with >2-fold downregulated expression (average) upon R-spondin withdrawal in intestinal organoid culture. Differential expression of genes 5 days after *Apc* deletion in mouse intestine (left), and upon R-spondin withdrawal in intestinal organoid culture at day 1, 2 and 3 (right). Scale bar in log₂ ratio is presented on the right (-2 to +2). Data were displayed using TreeView software. Red, upregulated; green, downregulated.

Gene name	log2
<i>SOX4</i>	3.1003
<i>7A5</i>	3.0196
<i>TGIF2</i>	2.7455
<i>ASCL2</i>	2.5956
<i>RNF43</i>	2.1319
<i>FOXA2</i>	2.0058
<i>FAM60A</i>	1.6961
<i>TCF7</i>	1.6735
<i>SOX9</i>	1.5932
<i>AXIN2</i>	1.2648
<i>FLJ21865</i>	1.2482
<i>ZNRF3</i>	1.2219
<i>FGFR4</i>	1.1189
<i>NKD1</i>	1.1129
<i>ZAK</i>	1.0894
<i>CYP39A1</i>	1.0663
<i>TFDP1</i>	0.9907
<i>KCNE3</i>	0.9572
<i>NEDD4</i>	0.8436
<i>LRCH1</i>	0.8373
<i>EFNA4</i>	0.7898
<i>SH3BP4</i>	0.7838
<i>SPAG4</i>	0.7717
<i>CKAP2</i>	0.7415
<i>GGH</i>	0.7175
<i>DACH1</i>	0.6997

Table 4. List of 26 genes with 1.5-fold upregulated expression after *Apc* deletion

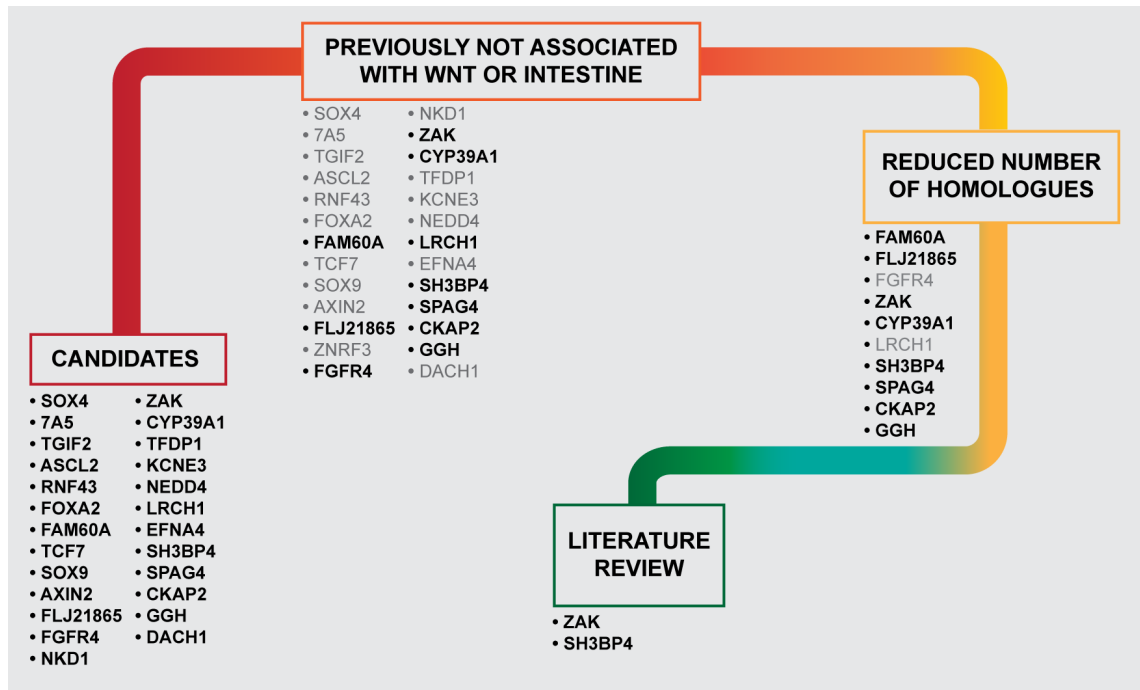


Figure 3.3 Schematic diagram of shortlisting strategy for putative Wnt regulators.

Genes in grey were excluded for not fulfilling the specified criteria. ZAK and SH3BP4 were the two top candidates.

3.2.3 Expression pattern of the selected candidate genes *Sh3bp4* and *Zak* in the intestine

To validate if ZAK and SH3BP4 are stem-cell expressing Wnt regulators in intestine, we first isolated crypt- and villus-enriched fractions from wild-type C57BL/6 mouse intestine and compared the mRNA expression of *Zak* and *Sh3bp4* between the two fractions using quantitative RT-PCR (qPCR) analysis. As expected, the established stem cell genes, *Lgr5* and *Olfm4*, were highly expressed in the crypt fraction, whilst the differentiated marker intestinal-type alkaline phosphatase (*Alpi*) was preferentially expressed in the villus fraction (Figure 3.4A). qPCR analysis also revealed nearly a 2-fold increase of both *Zak* and *Sh3bp4* mRNA levels in the crypt fraction when compared with the villus fraction. This fold change is similar to the stem cell-expressed genes, *Olfm4* and *Lgr5*.

To further demonstrate the spatial expression of *Sh3bp4* and *Zak*, RNAscope *in situ* hybridization (ISH) was performed in wild-type mouse intestinal tissues. Both *Sh3bp4* and *Zak* showed enriched expression in the bottom crypt stem cell

compartments in both small intestine and colon (Figure 3.4B). Co-staining with the stem cell marker, *Lgr5*, confirmed that both *Sh3bp4* and *Zak* are co-localised with *Lgr5* in the crypt base columnar cells (Figure 3.4C).

Next, we analysed the protein expression of ZAK and SH3BP4. Unfortunately, no commercially available antibodies were able to detect ZAK or SH3BP4 in mouse intestinal tissues using immunohistochemistry (IHC). We then performed Western blot analysis of the lysate prepared from villus-enriched versus crypt-enriched fractions from C57BL/6 mouse intestine (Figure 3.5A). Enriched expression of both ZAK and OLFM4 was detected in the crypt fraction, suggesting that the protein correlates with the mRNA expression data analysed by both qPCR and RNAscope ISH. Since no commercially available antibodies against SH3BP4 were able to recognise mouse protein, we generated knock-in (KI) mice with a FLAG-tag fused to the C-terminus of the SH3BP4 protein. The *Sh3bp4::FLAG* mice were generated by CRISPR/Cas9-mediated genome engineering and the design strategy is represented in Figure 3.5B. Two single-guide RNAs (sgRNAs) were designed to target the *Sh3bp4* locus at the 3'UTR region and create a double-strand break for homology direct repair. A single-stranded DNA oligo was designed with homology arms and two interspaced *FLAG* sequences. Three silent point mutations were created to avoid sgRNAs recognition at the donor DNA oligo. *Cas9* mRNA, the single-stranded DNA oligo and sgRNA 1 or sgRNA2 were microinjected into one-cell mouse embryos at pronuclear stage as previously described (Yang et al., 2014). Initially, all seven pups derived following sgRNA1 injection were wild-type. Subsequent sgRNA2 injections yielded 24 pups with only one showing incorporation of the donor *FLAG*-sequence KI. The insertion was confirmed by an increase of 57bp in the PCR size amplicon (Figure 3.5C). The correct orientation and insertion was further confirmed by sequencing. One allele contained the correct *FLAG*-sequence insertion, whereas the other allele was disrupted by non-homologous end joining (NHEJ). This founder mouse was then used for breeding, where germline transmission of the KI allele was observed in the F1 generation. Unfortunately, we were unable to detect FLAG expression by either Western blotting or IHC analysis in the heterozygous KI animal despite the correct sequence insertion. This could be due to either low protein abundance or incorrect protein folding. Homozygous animals are now being generated to see if the protein can be detected.

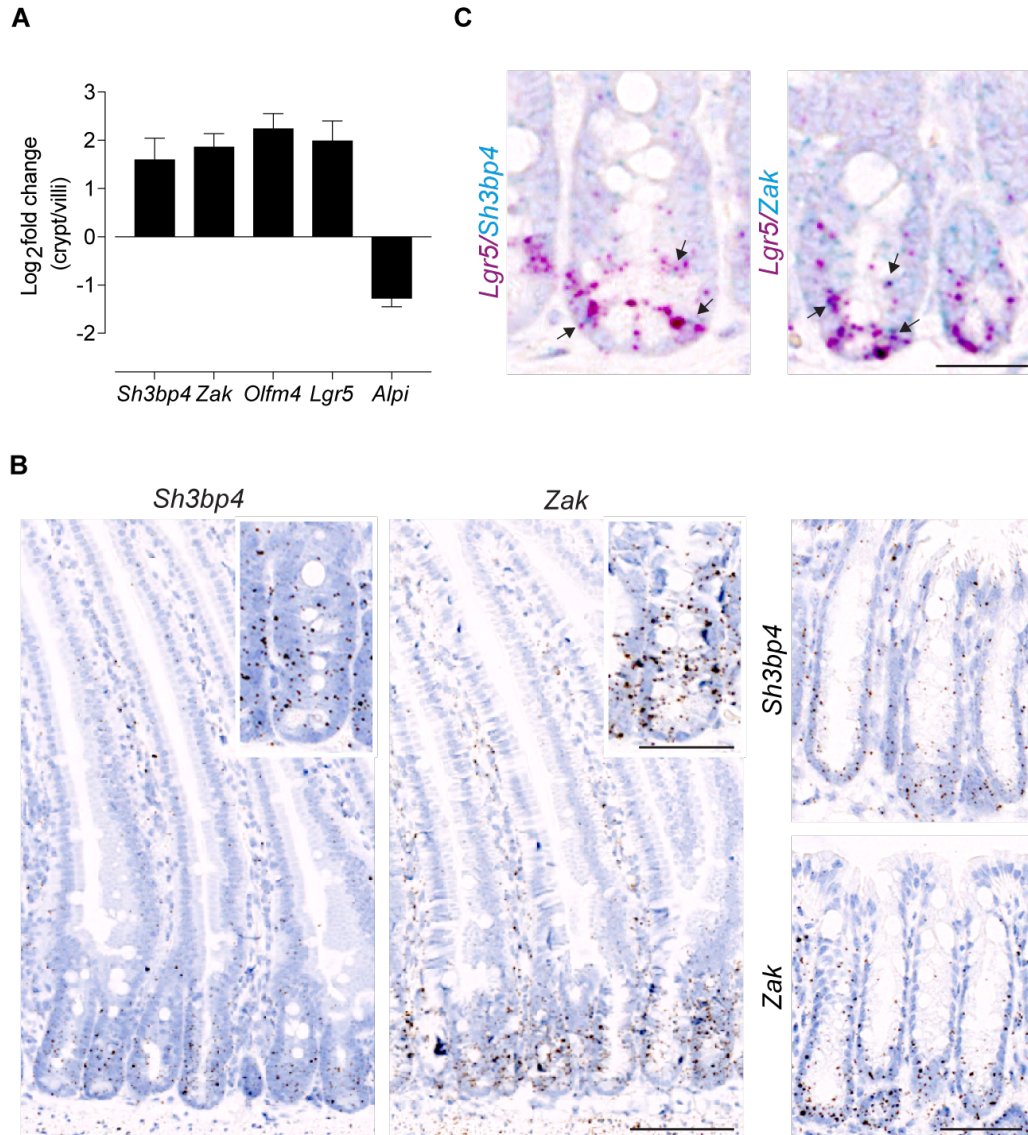


Figure 3.4 Expression pattern of the selected candidate genes, *Sh3bp4* and *Zak*, in the intestine.

(A) Q-PCR analysis showing *Sh3bp4* and *Zak* mRNA expression levels in crypt- and villus-enriched fractions isolated from C57BL/6J wild-type mouse intestine. Stem cell markers *Olfm4*, *Lgr5* and the enterocyte marker *Alpi* were used as a control for the two fractions (error bars represent averages \pm SD from 3 independent experiments, N=3). (B) Representative images of RNAscope ISH showing *Sh3bp4* and *Zak* gene expression in small intestine (left) and colon (right). (C) Representative images of RNAscope ISH showing co-localisation of *Sh3bp4* and *Zak* with *Lgr5* within the stem cell region. Arrows indicate co-localised staining. Scale bars, 100 μ m; scale bars of insets, co-expression images and colon, 50 μ m.

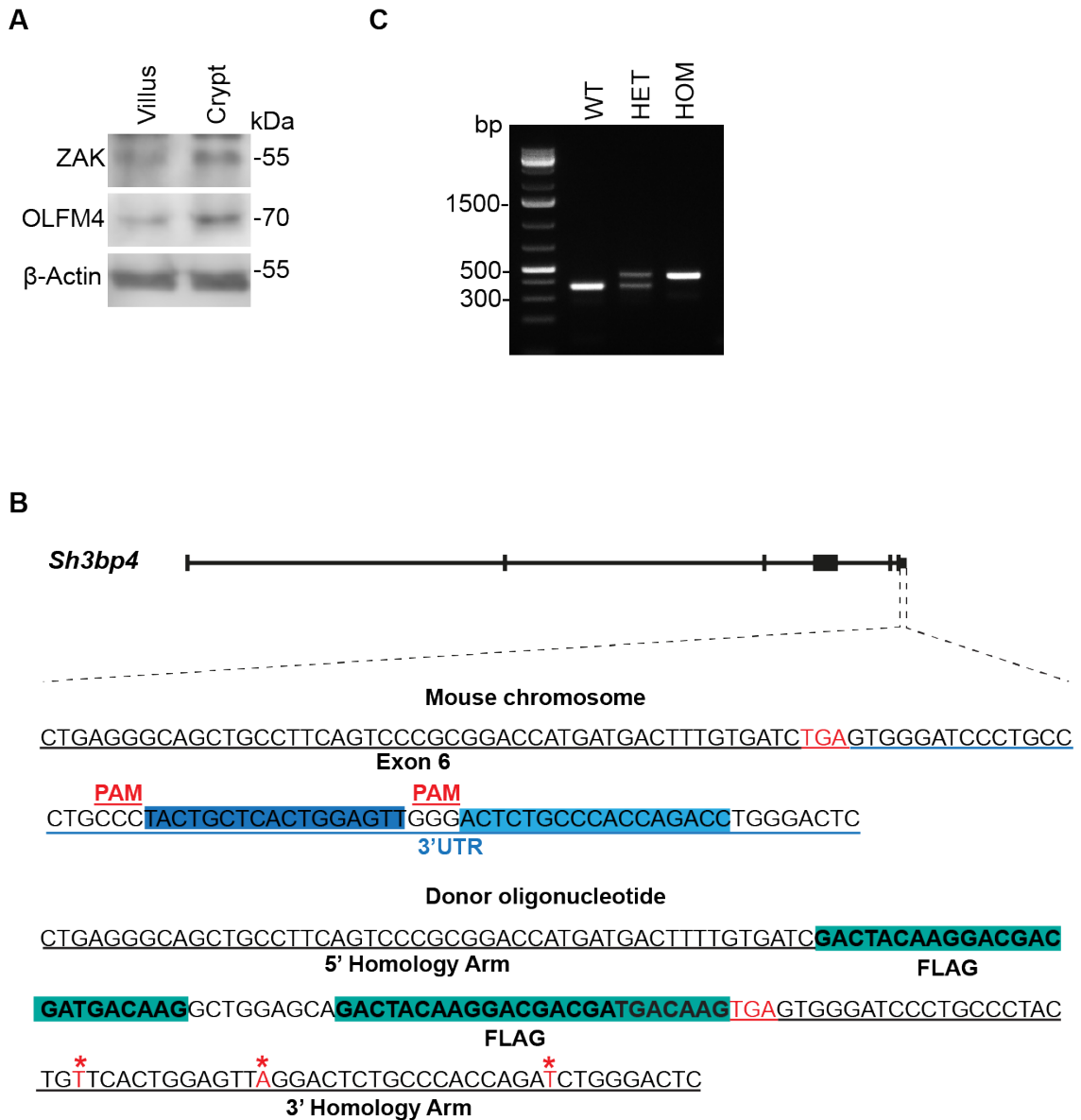


Figure 3.5 ZAK and SH3BP4 protein expression analysis.

(A) ZAK protein levels in the villus- and crypt-enriched fractions of wild-type mouse intestine. Western blot analysis showing enriched expression of ZAK and OLFM4 in crypts (representative image of $n=3$). (B) Schematic representation of the *Sh3bp4::FLAG* KI CRISPR/Cas9-targeting strategy. The top sequence shows the wild-type endogenous mouse chromosome sequence. The sgRNA1 is highlighted in dark blue and sgRNA2 highlighted in light blue. The bottom sequence shows the designed single-stranded DNA donor oligonucleotide. The 5' and 3' homology arms are indicated. Two *Flag* sequences are inserted before the stop codon interspaced with 9bp. Three point mutations are indicated with red asterisks. (C) PCR genotyping of the wild-type (WT), heterozygous (HET) and homozygous (HOM) *Sh3bp4::FLAG* KI animals. PCR amplicon of WT DNA yields a 323bp fragment, whereas the successful *Sh3bp4::FLAG* KI yields a larger 380bp amplicon.

3.2.4 Validation of *Zak* and *Sh3bp4* as Wnt target genes

To confirm if indeed *Zak* and *Sh3bp4* expression is increased upon Wnt signalling activation, we performed RNAscope in intestinal tissue from the *Apc^{min}* mouse model (Figure 3.6A). Both *Sh3bp4* and *Zak* showed enriched expression in *Apc^{min}* adenomas in comparison to adjacent normal tissue (Figure 3.6A). We then performed qPCR analysis in wild-type organoids and in organoids with *Apc* truncation. Briefly, APC mutation (Δ APC) was generated in organoids isolated from wild-type mouse using CRISPR-targeting, resulting in a truncated form of APC losing all the 20 amino acid repeats. These organoids persist as cysts as a result of hyperactivation of Wnt signalling (Novellasdemunt *et al*, accepted for publication). As expected, we observed a strong increase in *Zak* (~4-fold) and *Sh3bp4* (2-fold) expression (Figure 3.6B). In addition, we performed immunohistochemistry staining against SH3BP4 in human colorectal cancer samples (Figure 3.6C). As in mouse, SH3BP4 was exclusively expressed at the bottom of the crypt in normal adjacent tissue and is increased in tumour tissue. Together, this data corroborated the notion that both *Zak* and *Sh3bp4* expression is modulated by Wnt/ β -catenin signalling.

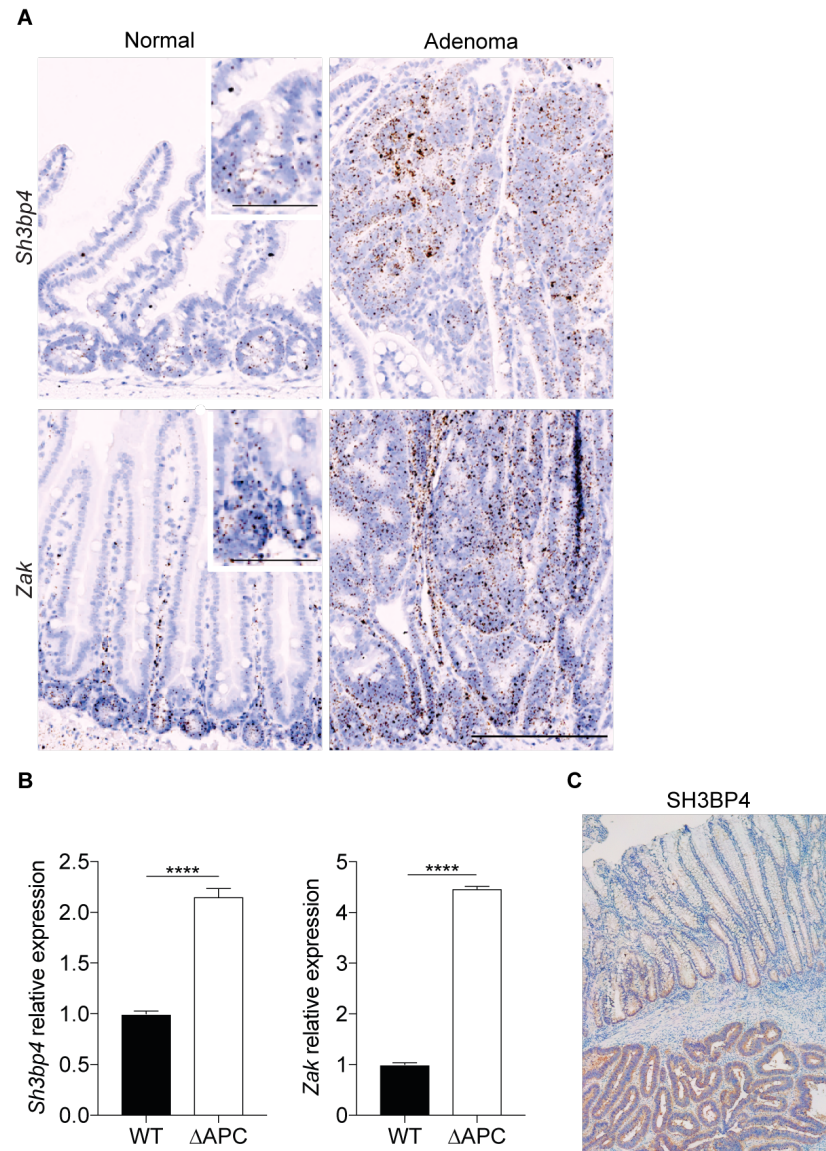
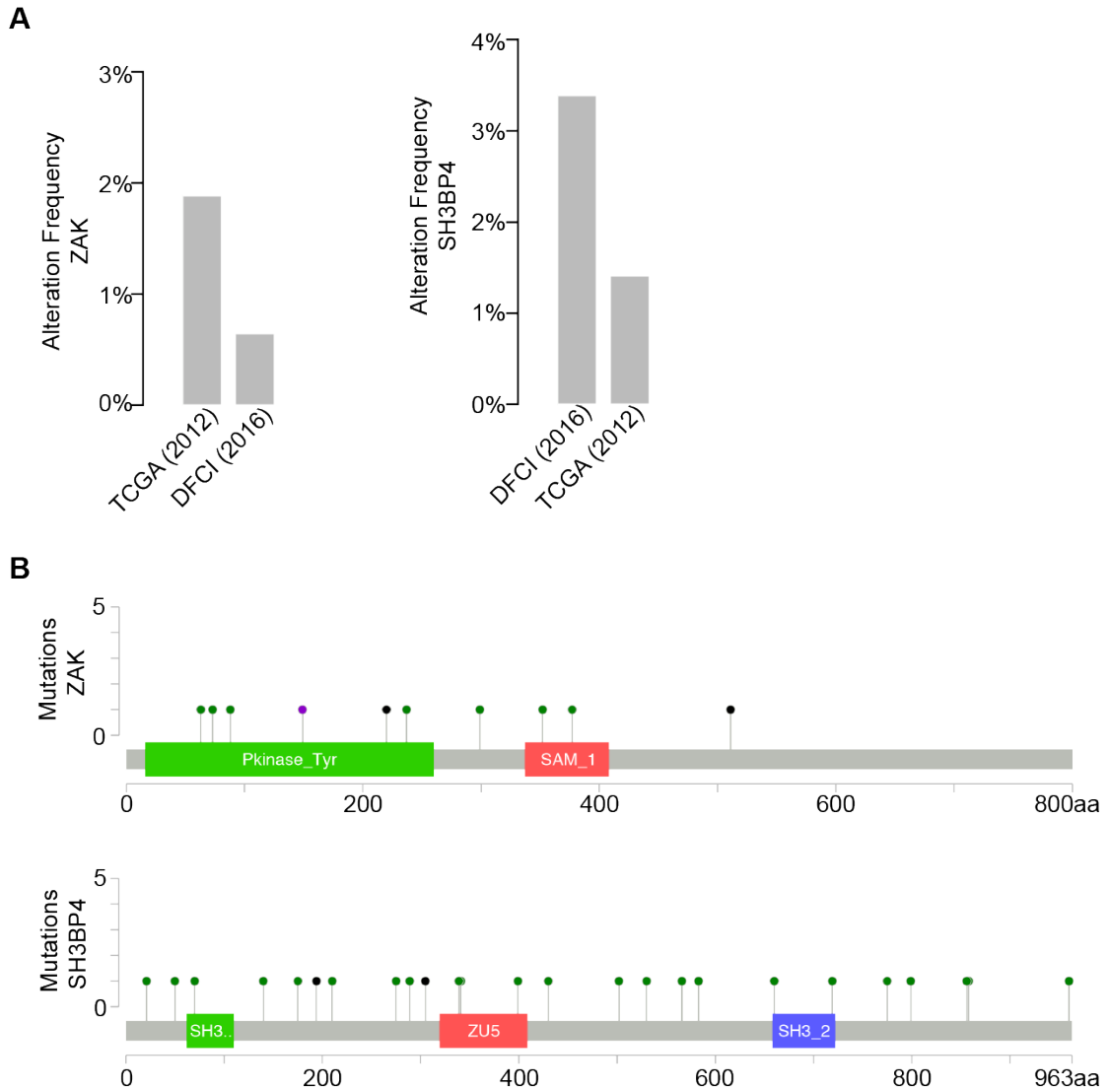


Figure 3.6 *Zak* and *Sh3bp4* expression are increased in *Apc^{min}* adenomas.

(A) Representative images of RNAscope ISH showing *Sh3bp4* and *Zak* gene expression in normal adjacent tissue and *Apc^{min}* adenomas (B) Quantitative RT-qPCR showing activation of *Sh3bp4* and *Zak* expression in wild-type organoids compared to *Apc*-mutated organoids. Expression data are presented as fold induction relative to GAPDH. Error bars represent averages \pm SD from 3 independent experiments (N=3). ***P< 0.001. (C) SH3BP4 staining in a human sample of CRC. Scale bars, 100 μ m, scale bars of insets, 50 μ m.

3.2.5 Mutations identified in public databases

Finally, we asked whether there was any genetic alteration of *ZAK* or *SH3BP4* reported in human CRC samples, which may provide further supportive evidence for their functional significance in tumourigenesis. The TCGA sequencing data showed that mutations of *ZAK* and *SH3BP4* are present, but only rarely with an alteration frequency of approximately 2% found in 212 CRC cases (Figure 3.7A). A more recent sequencing study from the Dana-Farber/Harvard Cancer Center with a larger cohort of CRCs (619 cases) showed a slightly higher *SH3BP4* mutation rate (3.4%, 21/619). Mutations of both *ZAK* and *SH3BP4* in CRCs are distributed across the whole gene without any hotspot (Figure 3.7B). The majority of the mutations identified are missense, although two truncating mutations are found in both *ZAK* and *SH3BP4*. Interestingly, the two truncating mutations identified in *ZAK* are located at mononucleotide repeats, c.659delA at A7 repeats and c.2296delG at G5 repeats. Interestingly, c.659delA was also reported in a gastric cancer sample, whilst another mutation c.657_658insA at the same A7 repeat region was found in a lung cancer patient. Similarly, the truncating mutation identified in *SH3BP4* by TCGA (c.581delT) is also located at T5 repeats. It is well known that MSI patients are prone to accumulate mutations at short nucleotide repeats due to the defective mismatch repair system. This may explain the association of downregulated expression of *ZAK* and *SH3BP4* with MSI CRCs. Further studies will be needed to characterise the functional significance of other specific missense mutations in CRC development.



3.3 Discussion

The advances in high-throughput sequencing over recent decades have provided valuable insights into the molecular events underlying CRC biology. It has become clear that CRC is a heterogeneous and complex disease. Multiple signalling pathways are involved in CRC initiation and progression. In particular, hyperactivation of the Wnt signalling pathway is the hallmark of CRC. In addition to the *APC* gene, many other Wnt signalling components are mutated in CRCs. Loss-of-function mutations in negative Wnt regulators, such as *AXIN2* and *RNF43*, also appear to be common in non-*APC* mutated CRC. Interestingly, most of these Wnt regulators also play a crucial role in normal intestinal homeostasis. Expression of these negative Wnt regulators in the intestinal stem cells further highlights the importance of precise control of the Wnt signalling at the “just-right” level via negative feedback mechanisms during normal tissue homeostasis. In this chapter, we focused on uncovering novel, potential Wnt regulators that are involved in both intestinal homeostasis and CRC development.

Expression profile analysis of the TCGA data identified a Stem-Cell/Wnt cluster that consists of several stem cell-expressed Wnt target genes. A number of them, such as *AXIN2*, *RNF43*, *ZNRF3*, *NKD* and *WIF*, have also been reported as negative Wnt regulators. Interestingly, genes in this cluster show differential expression between MSI and MSS-CRCs. In the majority of MSS-CRCs, Wnt activation upon APC loss results in general stem cell/Wnt target expansion (thus upregulated expression in the MSS-CRCs). Unlike in MSS tumours, MSI-CRCs might activate Wnt signalling through an alternative mechanism. For instance, mutations or epigenetic silencing of some Wnt inhibitors (e.g. *AXIN2*, *SFRP1-5*, *WIF1*) have been described in MSI tumours (Esteller et al., 2000; Fu et al., 2012; Koinuma et al., 2006; Murakami et al., 2014; Silva et al., 2014). We suggest that an overall downregulated expression in MSI-CRCs relative to MSS-tumours in the Stem-cell/Wnt cluster may represent an alternative Wnt-activating mechanism for non-*APC* mutated cancers by targeting the Wnt negative feedback mechanism at the stem cell region. Therefore, I hypothesised that many of the genes within this cluster are Wnt regulators exclusively expressed at the bottom of the crypt. Indeed, the genes within the cluster show relatively low expression in the normal cluster,

supporting the notion of restricted expression in normal intestinal tissue. It should be noted that other secretory Wnt inhibitors such as *SFRP1* and *DKK1-4* are expressed mostly in the mesenchyme rather than the stem cell region, and are thus located in different clusters.

Comparison of the gene expression profile in the Stem-cell/Wnt cluster with the *Rspo1* withdrawal and the *Apc* deletion signatures allowed us to further shortlist genes that respond to changes in Wnt signalling. Among the 26 shortlisted candidates, we have selected *ZAK* and *SH3BP4* for further downstream analysis based on literature review. *ZAK* is a member of the mixed-lineage kinases (MLKs) family (Gotoh et al., 2001). The role of *ZAK* in cancer development is controversial. The discrepancies between different studies could possibly be explained by the multiple roles of *ZAK* in activating distinct MAPK pathways in different cell types. Studies showing a direct role of the MLK family in Wnt signalling, and particularly *ZAK*, are limited and will be discussed in detail in the next chapter. *SH3BP4* has multiple motifs including SH3 domains, 3 Asn-Pro-Phe (NPF) motifs and ZU5 domains, suggesting multi-functional roles in various cellular processes. *SH3BP4* has been reported to regulate the mammalian target of rapamycin (mTOR) signalling (Kim et al., 2012b), whilst its role in Wnt/ β -catenin signalling or intestine has not been studied.

To study the role of *ZAK* and *SH3BP4* in intestinal homeostasis and cancer, we first tested whether the two putative Wnt target genes were expressed in the stem cells compartment. Both qPCR and RNAscope analysis confirmed that stem cells expressed the two genes in the base of intestinal crypts. Increased expression of *ZAK* and *SH3BP4* were also observed in the tumours of the APC^{min} intestinal tissues, supporting the microarray data. The results suggest that *ZAK* and *SH3BP4* are indeed Wnt targets expressed by stem cells. I propose that *ZAK* and *SH3BP4* may create a negative feedback loop to fine-tune the levels of Wnt signalling within the stem cell region during normal tissue homeostasis. Characterising the functional role of these Wnt inhibitors may help to shed light on new therapeutic strategies for treatment of CRCs. The role of *ZAK* and *SH3BP4* in Wnt signalling regulation and intestinal homeostasis will be analysed in detail in the following chapters.

Chapter 4. The ZAK kinase is a Wnt negative regulator expressed by stem cells

4.1 Introduction

The mitogen-activated protein kinase (MAPK) pathway consists of three sequentially activated protein kinases: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK (Chang and Karin, 2001; Nishida and Gotoh, 1993; Robinson and Cobb, 1997). It is a highly conserved signalling pathway that transduces a signal from the surface receptor to nuclear DNA transcription in response to different environmental stimuli via a three-step kinase cascade activation. ZAK (also known as MAP3K20) is a sterile- α -motif (SAM) and leucine zipper-containing protein that belongs to a subfamily of serine/threonine MAP3Ks known as mixed-lineage kinases (MLKs) (Gallo and Johnson, 2002; Gotoh et al., 2001; Liu et al., 2000a). Several studies have shown that ZAK is part of the signal transduction cascade that regulates extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (Cho et al., 2004; Gotoh et al., 2001; Liu et al., 2000a; Yang, 2002).

ZAK is expressed in many tissues including heart, skeletal muscle, lung, liver pancreas (Liu et al., 2000a), as well as in intestine as shown in chapter 3. ZAK has two alternative splicing isoforms: ZAK α and ZAK β (Gotoh et al., 2001; Liu et al., 2000a). ZAK α , the longer isoform, encodes an 800 amino acid protein that contains a kinase catalytic domain, a leucine zipper domain (LZ), and a SAM (Figure 4.1A). The kinase domain of ZAK is encoded by exon 2, consists of 245 amino acids (from 16-260) and is responsible for serine/threonine phosphorylation of its substrates (Liu et al., 2000a). The LZ domain can mediate the homodimerisation of many MLKs members and is critical for ZAK's autophosphorylation (Nihalani et al., 2000). The SAM domain consists of 74 amino acids (from amino acid 336 to 410) and is exclusively present in the longer isoform, ZAK α . Some studies have demonstrated that the SAM domain functions as a protein interaction domain (Stapleton et al., 1999). ZAK β consists of 455 amino acids and shares the same N-terminal protein sequence but terminates shortly after the catalytic domain. This shorter isoform

contains a conserved, unique C-terminal extension of 124 amino acids, which includes an extended acidic patch (Gotoh et al., 2001; Liu et al., 2014b).

ZAK has been implicated in cancer development however, its role remains controversial. Several studies have proposed ZAK to be an oncogene. Overexpression of ZAK in skin epidermal cells resulted in neoplastic cell transformation when injected subcutaneously into athymic nude mice (Cho et al., 2004). In addition, knockdown of ZAK inhibits proliferation in gastric cell lines (Liu et al., 2014b). On the other hand, overexpression of ZAK resulted in cell death in a hepatoma cell line (Liu et al., 2000a) and inhibited cell proliferation in embryonic fibroblasts (Yang, 2002) and lung cancer cells (Yang et al., 2010). The discrepancy between these results could be due to the diverse roles of ZAK in activating distinct MAPK pathways in different cell types. In addition, ZAK has also been reported to be expressed in different subcellular compartments in various cell types (Choi et al., 2005; Gotoh et al., 2001). Several studies have further suggested that the two isoforms may have distinct biological functions (Gotoh et al., 2001; Liu et al., 2014b).

Some studies have described an association between ZAK and Wnt signalling and CRC development. Firestein and colleagues have performed a RNAi-based loss-of-function screen which identified ZAK as one of the top nine candidates to modulate both β -catenin-dependent transcription (in DLD1 cells) and colon cancer cell proliferation (HCT116 cells) (Firestein et al., 2008). However, only CDK8, one of the top candidates, was further characterise (Firestein et al., 2008) and the mechanistic role of ZAK in has not been studied. Furthermore, a genome-wide sequencing study showed significant upregulation of the ZAK α isoform in gastric cancers as well as in CRCs (Liu et al., 2014b). In this study, Liu and colleagues have analysed the effect of ZAK in several cancer-related signalling reporters and have shown that ZAK is capable of modulating the transcription activity of AP1, NF κ B and particularly, the Wnt signalling TCF/LEF reporter assay (Liu et al., 2014b). On the other hand, ZAK has been reported to regulate expression of the SOX protein (Suzuki et al., 2012). Interestingly, increasing evidence suggests that many SOX proteins can physically interact with β -catenin and modulate β -catenin/TCF transcriptional activity (Kormish et al., 2010).

As discussed in Chapter 1, crosstalk between Wnt signalling and other pathways is important for CRC progression. Given the potential function of ZAK in

activating different MAPK pathways, it is worth discussing the crosstalk between different MAPKs and Wnt/ β -catenin signalling in intestinal homeostasis and cancer.

p38 MAPK is often deregulated in cancers but a molecular understanding of how p38 family members function either as tumour suppressors or oncoproteins remains unclear. Previous studies showed that p38 negatively regulates cell cycle progression (Hui et al., 2007) and induces apoptosis (Cai et al., 2006), supporting the notion of a role for p38 as tumour suppressor. In addition, p38 activation has been shown to trigger differentiation and a less transformed phenotype in colon cancer cell lines (Houde et al., 2001; Ordonez-Moran et al., 2008). In an *in vivo* mouse model, downregulation of p38 in intestinal epithelial cells resulted in increased proliferation and reduced enteroendocrine and goblet cell differentiation in colon (Gupta et al., 2014). On the other hand, another study showed that p38 can directly phosphorylate and inactivate GSK3 β , which could potentially result in an accumulation of β -catenin (Thornton et al., 2008).

A study from Jeong and colleagues has shown evidence of crosstalk between ERK and Wnt/ β -catenin signalling in the intestine. The study revealed that Wnt/ β -catenin signalling activation promotes both β -catenin and Ras stabilisation through the phosphorylation-ubiquitination axis mediated by the β -catenin destruction complex (Jeong et al., 2012). The data suggests that aberrant Wnt activation promotes intestinal tumorigenesis by synergistic activation of both β -catenin and RAS/ERK signalling. The role of MEK1/Shp2/MAPK signalling in controlling intestinal stem cell fate has also been reported; Heuberger and colleagues showed that the level of MAPK signalling determines the choice between goblet and Paneth cell fate (Heuberger et al., 2014). MAPK inhibition enhances Wnt/ β -catenin signalling and promotes Paneth cells differentiation, whereas high MAPK activity or inhibition of Wnt signalling favours goblet cell differentiation. The study further showed that MAPK inhibition promotes preferential expression of the longer TCF4 isoform (Heuberger et al., 2014), which consists of a binding site for the transcription factor carboxyl-terminal binding protein (CtBP) and a C-terminal DNA binding domain (Weise et al., 2010). A more recent study using the organoid culture system also showed that ERK/MAPK signalling, downstream of EGFR, is required for the proliferation of *Lgr5*⁺ cells, whereas inhibition of MAPK/Wnt/Notch signalling is required for enteroendocrine cell differentiation (Basak et al., 2016).

The crosstalk between JNK and Wnt signalling is less characterised. JNK has been shown to antagonise Wnt/ β -catenin signalling in *Xenopus* embryos, whilst activation of JNK signalling in mammalian cells reduces β -catenin-mediated gene expression by targeting its nuclear export (Liao et al., 2006). MAP3K1 has previously been shown to modulate JNK activity via MMK4 (Yan et al., 1994). Interestingly, MAP3K1 was also found to modulate Wnt signalling activity by interacting with AXIN1 (Sue Ng et al., 2010), although the substrate of MAP3K1 within the Wnt cascade has not yet been revealed.

Taken together, it is still unclear how the crosstalk between MAPK and Wnt signalling regulates intestinal homeostasis and tumorigenesis. As discussed earlier, ZAK has been implicated in Wnt signalling and cancer, although its mechanistic role has not been studied. Our current data suggest that the stem cell/crypt-expressing ZAK is likely a Wnt target gene that may serve as a feedback regulator of the pathway. In this chapter, I explore the role of ZAK in Wnt signalling and intestinal homeostasis. In particular, I aim to characterise whether the Wnt regulatory role of ZAK is dependent or independent of MAPK activity.

4.2 Results

4.2.1 ZAK is a negative regulator of Wnt/ β -catenin signalling

In the previous chapter, we identified ZAK as a putative Wnt regulator localised in the Stem-Cell/Wnt cluster. Expression of *Zak* is restricted to the Wnt-active crypt compartment, and enriched expression in *Apc* mutated tumours and organoids further suggest that *Zak* is a Wnt transcriptional target. Moreover, we have confirmed the co-expression of *Zak* and *Lgr5* in intestinal crypts, to study the role of ZAK in regulating Wnt/ β -catenin signalling, HEK293T cells with intact Wnt signalling cascade, were used for initial gain-of-function and loss-of-function studies. Deletion of ZAK in HEK293T cells was performed using the CRISPR/Cas9 gene editing system via non-homologous end-joining (NHEJ). Single-guide RNA (sgRNA) targeting exon2, which contains the start codon and the kinase domain, was designed to mutate both ZAK isoforms. The CRISPR-targeted clone (Δ ZAK) was generated and homozygous frameshift mutations were confirmed by sequencing

(Figure 4.1B). One allele contains a 189bp insertion that results in a truncated product of 42 amino acids. The second allele harbours a 10bp deletion also causing an earlier truncation at residue 88. Nucleotide sequences and predicted amino acid sequences are shown in appendix 1. The TCF-TopFlash luciferase reporter assay was used to measure Wnt/TCF transcriptional activity in the subsequent experiments (see chapter 2 for more details).

As shown in Figure 4.1C, depletion of ZAK resulted in more than a 2-fold increase in Wnt3A-induced TCF-TopFlash reporter activity in HEK293T cells. In the reverse experiment, overexpression of wild-type ZAK significantly suppressed Wnt3A-induced TCF-TopFLASH reporter activity, suggesting that ZAK is a negative Wnt regulator (Figure 4.1D). To address whether the kinase function of the protein is responsible for inhibiting Wnt signalling, we generated a kinase-dead mutant version of ZAK by site-directed mutagenesis at lysine 45, the ATP binding site of the kinase (ZAK^{K45A}) (Gross et al., 2002). In contrast to the wild-type protein, expression of ZAK^{K45A} kinase mutant failed to inhibit Wnt3A-induced TCF-TopFLASH reporter activity, suggesting that the kinase activity of ZAK is indeed required to inhibit Wnt/ β -catenin signalling in mammalian cells. To further confirm the negative Wnt regulatory role of ZAK, we performed a rescue experiment by overexpressing ZAK in the Δ ZAK mutant cells. As expected, the increase in Wnt3A-induced TCF-TopFLASH reporter activity upon ZAK depletion was readily rescued by expression of wild-type ZAK protein (Figure 4.1E). On the other hand, expression of ZAK^{K45A} kinase mutant failed to inhibit the upregulated Wnt signalling caused by ZAK deletion. The TCF-reporter data was further confirmed by western blot analysis using an antibody against active β -catenin (non-phosphorylated form) (Figure 4.1F). Consistent with the TCF reporter data, β -catenin level was upregulated in Δ ZAK compared to wild-type HEK293T cells (lane 1 and lane 2), whilst the upregulated β -catenin level was inhibited by expression of wild-type ZAK (lane 3) but not ZAK^{K45A} mutant (lane 4). Together, the data support the notion that the ZAK kinase plays an inhibitory role in Wnt/ β -catenin signalling.

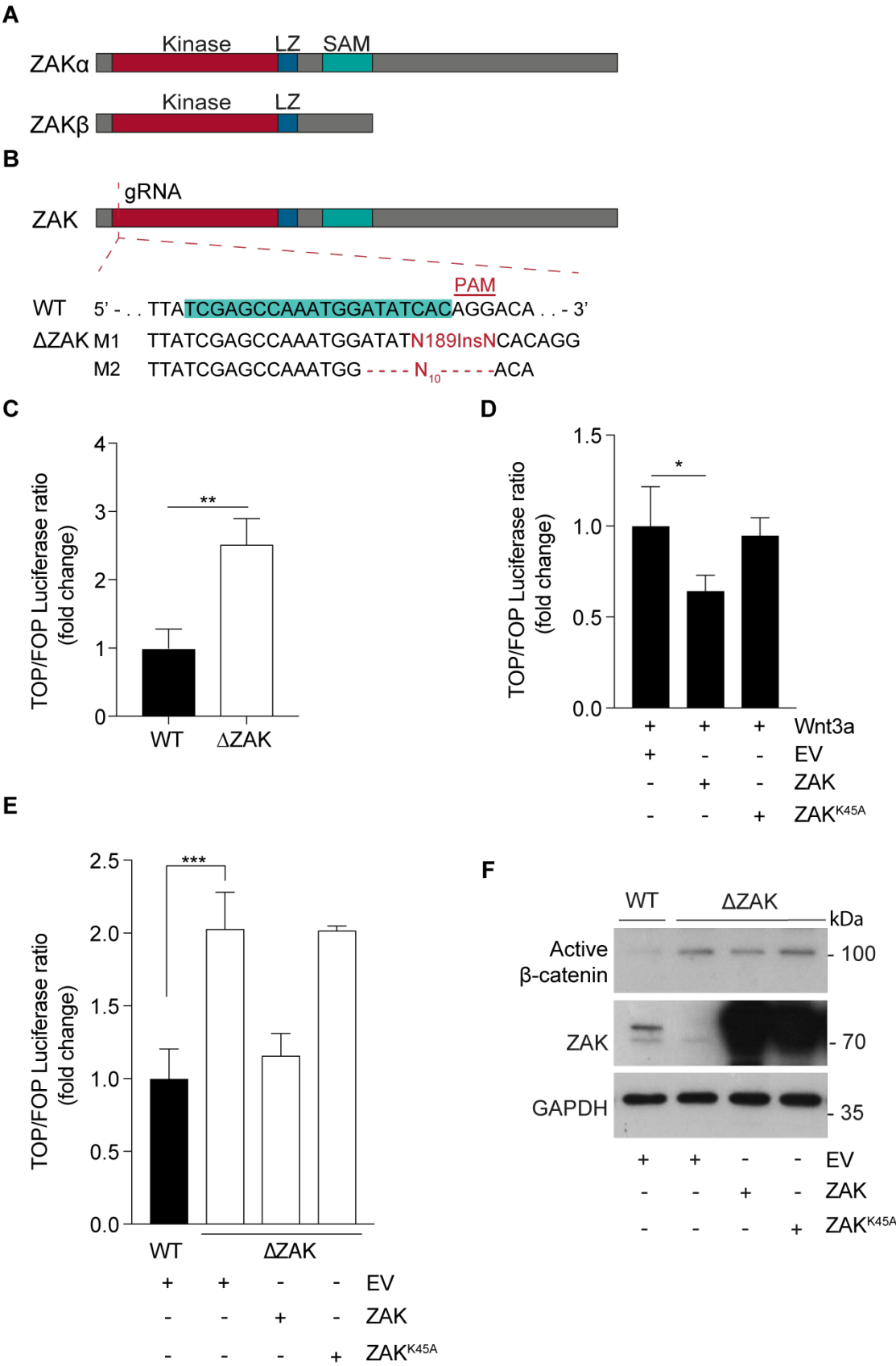


Figure 4.1 ZAK inhibits Wnt/ β -catenin signalling.

(A) Domain structure of ZAK α and ZAK β isoforms. ZAK α is the longer isoform and encodes an 800 amino acids protein. ZAK β is the shorter isoform and is composed by 455 amino acids. Both isoforms contain a kinase catalytic domain (in red) encoded by exon 2. The kinase domain consists of 245 amino acids and is responsible for serine/threonine phosphorylation. The leucine zipper domain (LZ – in blue, 22 amino acids) is also shared by the two isoforms and it can mediate the homodimerisation of many MLKs. The sterile- α -motif (SAM –light blue) consists of 74 amino acids and it is only present in ZAK α . This domain seems to function as a protein-protein interaction domain. Both isoforms share the N-terminus but ZAK β has a unique C-terminal region, which includes an extended acidic patch. (B) Schematic representation of the CRISPR/Cas9 targeting at *Zak* exon 2. Frameshift mutations detected in the selected HEK293T clone, M1 and M2, are indicated. (C) TCF-TopFlash reporter assay showing an increase in Wnt signalling activity in Δ ZAK versus WT HEK293T cells treated with Wnt3A condition medium. (D) Relative TCF-TopFlash reporter activity in Wnt3A-induced HEK293T cells with co-expression of the indicated plasmids. (E) Relative TCF-TopFlash reporter activity in Wnt3A-induced wild-type HEK293T cells or Δ ZAK cells co-expressing the indicated plasmids. (F) Western blot analysis of samples obtained from (E) using the indicated antibodies. The Top and Fop signals were normalised to the renilla luciferase signal and average from three independent experiments are shown (N=3). The error bars represent \pm SD. ***P \leq 0.001, **P \leq 0.01 and *P \leq 0.05.

4.2.2 The Wnt negative regulator ZAK functions downstream of β -catenin phosphorylation/ubiquitination

To dissect the mechanistic role of ZAK in Wnt/ β -catenin signalling regulation, we investigated ZAK's potential to inhibit the signalling cascade at different subcellular levels (membrane receptor and cytoplasmic destruction complex), using various Wnt activation models. A mutant form of LRP6 lacking the extracellular domain (Δ N-LRP6) has been reported as a constitutively activated Wnt receptor (Liu et al., 2003). The PPPSPxS motif of Δ N-LRP6 is constitutively phosphorylated, generating a persistent docking site for AXIN that mimics the membrane recruitment of the AXIN-destruction complex upon Wnt induction. Similar to the Wnt3A induction experiment, expression of wild-type ZAK, but not the ZAK^{K45A} mutant, significantly inhibited Δ N-LRP6-induced Wnt/ β -catenin activation (Figure 4.2A). The result suggests that ZAK inhibits Wnt signalling downstream of LRP6 phosphorylation. Next, we tested whether ZAK can inhibit Wnt signalling activated at the destruction complex level using two different models. First, we used a constitutively active form of β -catenin in which serine 33 was mutated to tyrosine (β CatS33Y), destroying the GSK3 β -mediated phosphorylation site. Similarly, whilst the wild-type ZAK was able to suppress β CatS33Y-induced Wnt activation, the ZAK^{K45A} mutant failed to execute such inhibition (Figure 4.2B). In a second model, we used an APC-truncated isogenic mutant line generated for a separate project in the lab. Briefly, an APC mutation at 1225 amino acids was generated in HEK293T cells using CRISPR-targeting (HEK293T Δ APC), resulting in a truncated form of APC losing all the 20 amino acid repeats. Data from our lab confirmed that the Δ APC mutant significantly activates Wnt signalling (>1000 fold TCF-TOPFlash activity) by suppressing β -catenin ubiquitination despite normal phosphorylation (Novellasdemunt et al., 2017). Once again, expression of wild-type ZAK, but not the ZAK^{K45A} kinase mutant, inhibited the Wnt/ β -catenin signalling in HEK293T Δ APC (Figure 4.2C). Together, the data support a model in which ZAK inhibits Wnt/ β -catenin signalling downstream of β -catenin phosphorylation and ubiquitination. Interestingly, immunofluorescent staining of FLAG-ZAK expression in HEK293T cells revealed an exclusive cytoplasmic localisation of the protein (Figure 4.2D). The absence of the protein in the nucleus suggests that ZAK may function upstream of β -catenin/TCF transcription. Further

investigation will be needed to characterise the precise role of ZAK in the Wnt signalling cascade.

As mentioned earlier, ZAK has been reported to activate several MAPK pathways, in particular MEK1/2, JNK, and p38. To study if the Wnt inhibitory role of ZAK is dependent on MAPKs activation, I repeated the experiments using various MAPK inhibitors in one of the Wnt-activating models (HEK293T Δ APC). If ZAK's negative Wnt regulatory role is mediated via a specific MAPK activation, treatment of the appropriate MAPK inhibitor should rescue the Wnt inhibitory effect observed in the HEK293T Δ APC cells. Wild-type and the kinase-mutant ZAK were expressed in HEK293T Δ APC cells treated with individual MAPK inhibitors, including MEK1/MEK2 inhibitor (SCH772984), JNK inhibitor (SP600125), p38 inhibitor (SB202190), and ERK5 inhibitor (XDM8-92) (Figure 4.3). Interestingly, treatment with various MAPK inhibitors did not affect the capacity of wild-type ZAK in suppressing Wnt/ β -catenin signalling, suggesting that the Wnt regulatory role played by ZAK is independent of MAPK signalling. Further studies of combined treatment of various MAPK inhibitors will be important to rule out any crosstalk effect of different MAPK pathways on Wnt signalling. In summary, our data suggest that ZAK inhibits Wnt/ β -catenin signalling downstream of β -catenin phosphorylation and ubiquitination, independently of either MEK1/2, JNK, p38 or ERK5 pathways alone.

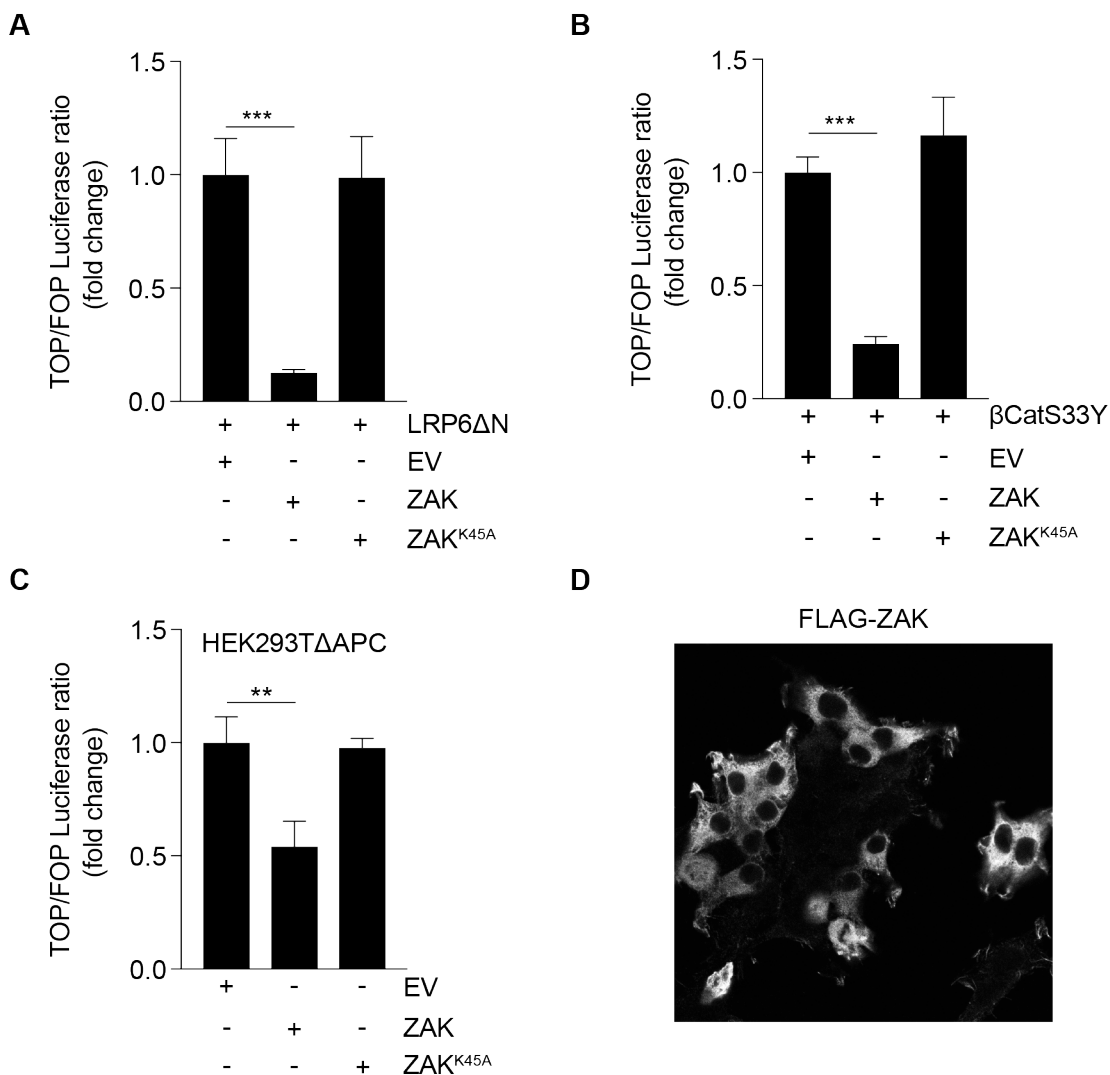


Figure 4.2 ZAK inhibits Wnt signalling downstream of β -catenin phosphorylation and ubiquitination.

(A) Relative TCF-TopFlash reporter activity induced by LRP6 expression construct lacking the extracellular domain (Δ N-LRP6). Δ N-LRP6 was co-expressed with empty-vector (EV), ZAK or ZAK^{K45A} in HEK293T cells. (B). EV, ZAK or ZAK^{K45A} constructs were co-expressed with the constitutively active β -catenin mutated at serine 33 (β CatS33Y). ZAK is capable of suppressing TCF-TopFlash reporter induced by β CatS33Y. (C) HEK293T cells containing a truncated form of the APC protein (HEK293T Δ APC) were transfected with EV, ZAK or ZAK^{K45A}. ZAK but not ZAK^{K45A} suppresses TCF-TopFlash luciferase reporter signal. (D) Immunofluorescent staining of FLAG-ZAK using an antibody against FLAG epitope showing exclusive cytoplasmic localisation. The Top and Fop signals were normalised to the renilla luciferase signal and average from three independent experiments are shown (N=3). The error bars represent \pm SD. ***P \leq 0.001 and **P \leq 0.01.

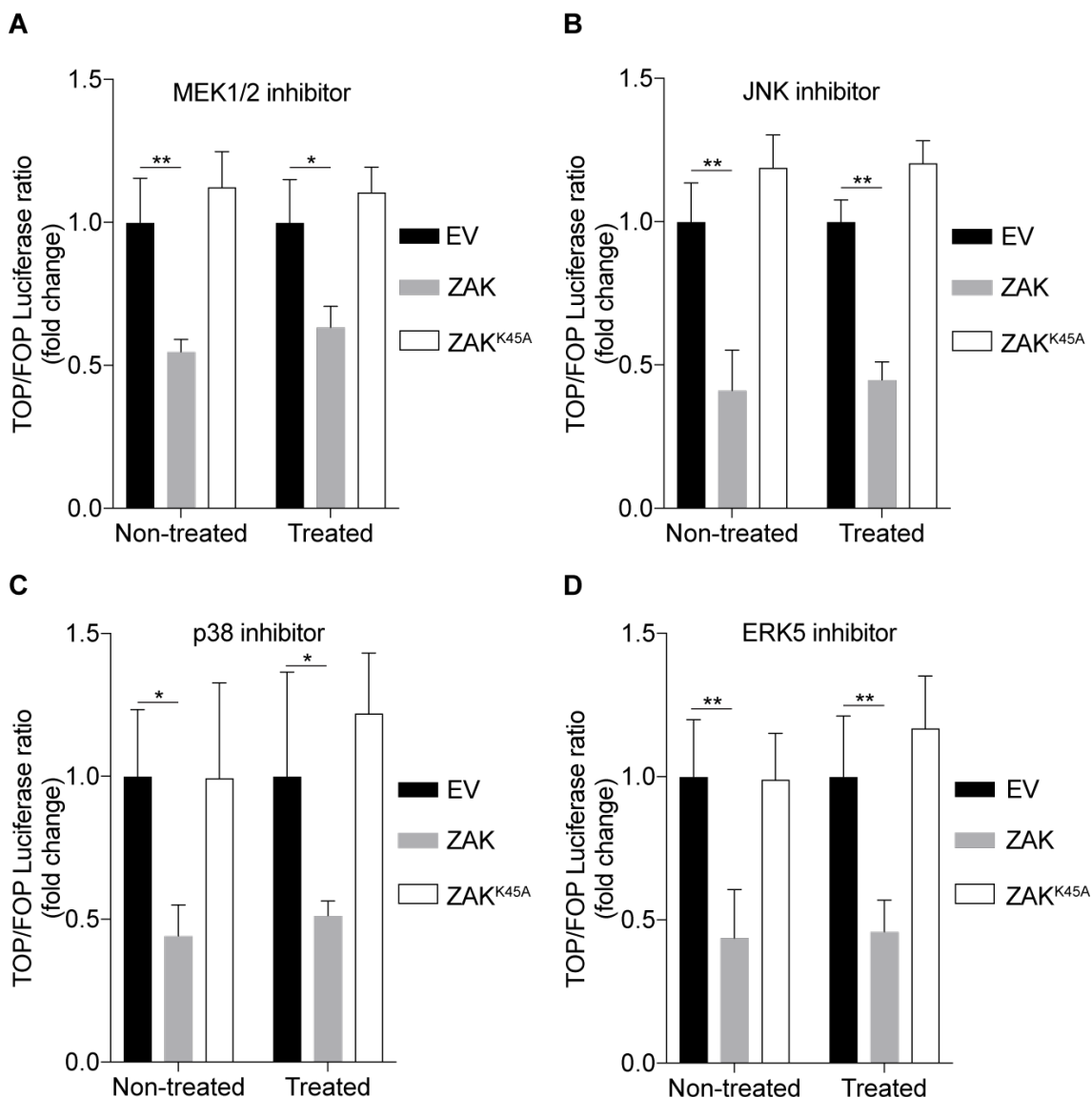


Figure 4.3 The Wnt inhibitory role of ZAK is independent of MAPK signalling.

Relative TCF-TopFlash reporter activity of HEK293TΔAPC cells expressing empty vector, ZAK or ZAK^{K45A} construct treated with the indicated MAPK inhibitors for 24hours: (A) 10 μ M of MEK1/2 inhibitor (SCH772984), (B) 5 μ M of JNK inhibitor (SP600125), (C) 5 μ M of p38 inhibitor (SB202190) and (D) 10 μ M of ERK5 inhibitor (XDM 8-92). The Top and Fop signals were normalised to the renilla luciferase signal and average from three independent experiments are shown (N=3). The error bars represent \pm SD. **P \leq 0.01 and *P \leq 0.05.

4.2.3 *Zak*-null mouse characterisation

To further study the biological relevance of ZAK in intestinal homeostasis, I proceeded to generate *Zak*-null mice for functional studies *in vivo*. CRISPR/Cas9 genome editing technology has been shown to be efficient in generating knockout mice (Wang et al., 2013). Since the sgRNA sequence previously used to target ZAK in HEK293T cells in section 4.2 was proven to be efficient, this was also used for mouse targeting as the sequence was shared between human and mouse. Both mRNA of the sgRNA and Cas9 were microinjected into the pronuclear stage one-cell mouse embryos collected from C57BL/6xCBA(F1) background mice (see chapter 2 for detailed information). From 3 recipient females, 46 pups were obtained in total. MiSeq analysis confirmed that all 46 pups were positive for five different indel mutations generated by NHEJ. Two females and one male carrying the founder mutations were inter-crossed to obtain immediate *Zak*-null mutants in F1 generation. Four mutated alleles were successful germline transmitted and these are indicated in Figure 4.4A. Mutation 1 (M1) and 4 (M4) harbour a 10bp deletion, whilst mutations 2 (M2) and 3 (M3) show 1bp and 5bp deletions respectively. All mutations were predicted to yield a truncated form of the ZAK protein, which we confirmed by Western blot in intestinal epithelium lysates from heterozygous *Zak*^{-/-} F1 mice (Figure 4.4B).

The *Zak*^{-/-} mice developed normally and no obvious physical or behaviour phenotype was observed. As mentioned above, ZAK is expressed in heart, muscle, lung, liver and pancreas (Liu et al., 2000a). None of these organs have shown macroscopic differences when compared to the wild-type animals. However, we have shared *Zak*^{-/-} animals with two different laboratories that have reported a phenotype in muscle morphology and in endoplasmic reticulum function. These phenotypes are now being explored by our collaborators to better understand the physiological significance of ZAK protein in these contexts.

To study the role of ZAK in intestinal homeostasis, intestinal tissues from 4-month-old wild-type (WT) and *Zak*^{-/-} mice were isolated for histological analysis. Surprisingly, no gross morphological differences were observed in *Zak*^{-/-} intestine compared to WT animals (Figure 4.4C). Intestinal villus and crypt length was measured in all intestinal regions for comparison and no differences were observed

between WT and *Zak*^{-/-} mice in either duodenum, jejunum, ileum or colon (Figure 4.4D-E). To determine the impact on intestinal homeostasis in older mice, a cohort was aged for one 1 year and *Zak*^{-/-} and WT animals were analysed. Similar to the younger animals, histological analysis of the intestinal tissues from one-year-old WT and *Zak*^{-/-} mice showed no apparent morphological differences (Figure 4.4F).

For better characterisation, proliferation and differentiation potential of the intestinal epithelium in *Zak*^{-/-} mice was further analysed. No significant differences in stem cell proliferation was observed between WT and *Zak*^{-/-} intestines using Ki67 staining or EdU (Figure 4.5A and B). An average of 10±1 EdU⁺ cells/crypt was observed in *Zak*^{-/-} and WT intestines (Figure 4.5B). Intestinal epithelial turnover rate was further analysed by 5-ethynyl-2'-deoxyuridine (EdU) tracing. Mitotically active proliferative cells were chemically labelled with EdU and traced for 3 days. Intestinal tissues of WT and *Zak*^{-/-} mice were collected 2 hours, 1 day and 3 days after EdU injection. The EdU-labelled cells appeared to migrate towards the villi at similar rates between *Zak*^{-/-} and WT intestines, indicating that loss of ZAK did not affect proliferation or turnover rate of the intestinal epithelium (Figure 4.5C). Immunostaining of the Wnt target SOX9 did not reveal any major differences either, suggesting that Wnt/β-catenin signalling was not affected in *Zak*^{-/-} animals during normal homeostasis (Figure 4.5D). Analysis of the two differentiation markers lysozyme (Paneth cells) and periodic acid-Schiff⁺ (PAS) (goblet cells) also showed no difference in lineage differentiation in *Zak*^{-/-} intestines (Figure 4.5E-G). An average of 6±1 PAS⁺ cells/crypt was observed in both WT and *Zak*^{-/-} intestines (Figure 4.5G). Together, the results suggest that ZAK is dispensable for normal intestinal homeostasis.

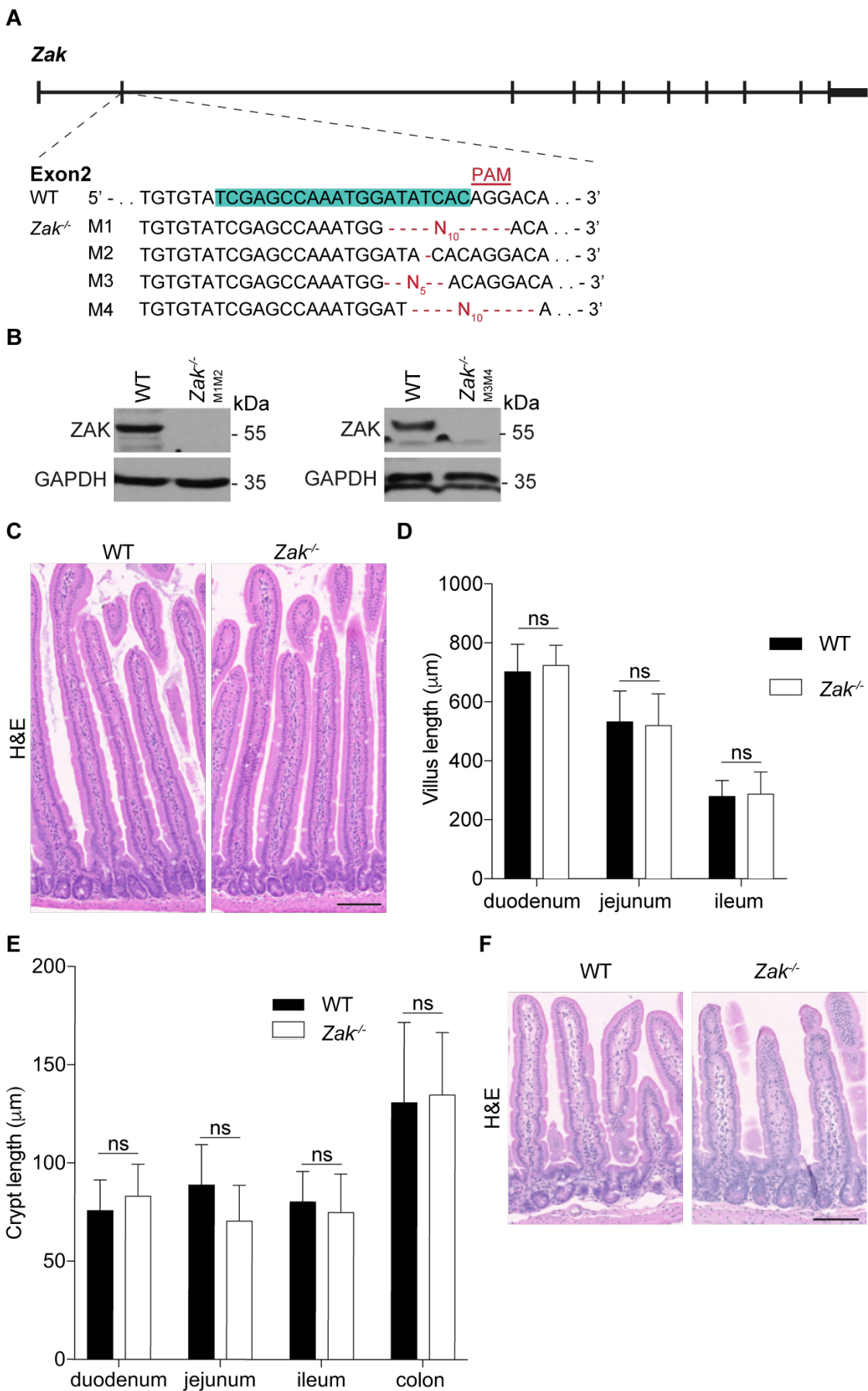


Figure 4.4 Generation of *Zak*^{-/-} mice by CRISPR/Cas9 genome editing.

(A) Schematic representation of the CRISPR/Cas9 targeting strategy of *Zak* at exon 2. gRNA used is highlighted in blue and the PAM sequence is also indicated. Four transmitted frameshift mutations genotypes (M1-M4) found in F1 *Zak*^{-/-} mice are indicated. (B) Western blotting shows the absence of ZAK protein in the whole intestinal tissue lysates obtained from WT and *Zak*^{-/-} mice. (C) Representative H&E image of duodenum section from 3-month-old WT and *Zak*^{-/-} mice. (D) Measurement of villi length in WT and *Zak*^{-/-} animals in all regions of small intestine. 30 villi/region were measured and means \pm SD of 3 mice per group are shown. ns - non-significant ($p>0.05$). (E) Measurement of crypt length in all regions of small and large intestine. 30 crypts/region were measured and means \pm SD of 3 mice per group are shown. (F) Representative H&E image of small intestine section from 1-year-old WT and *Zak*^{-/-} mice. Scale bars, 100 μ m.

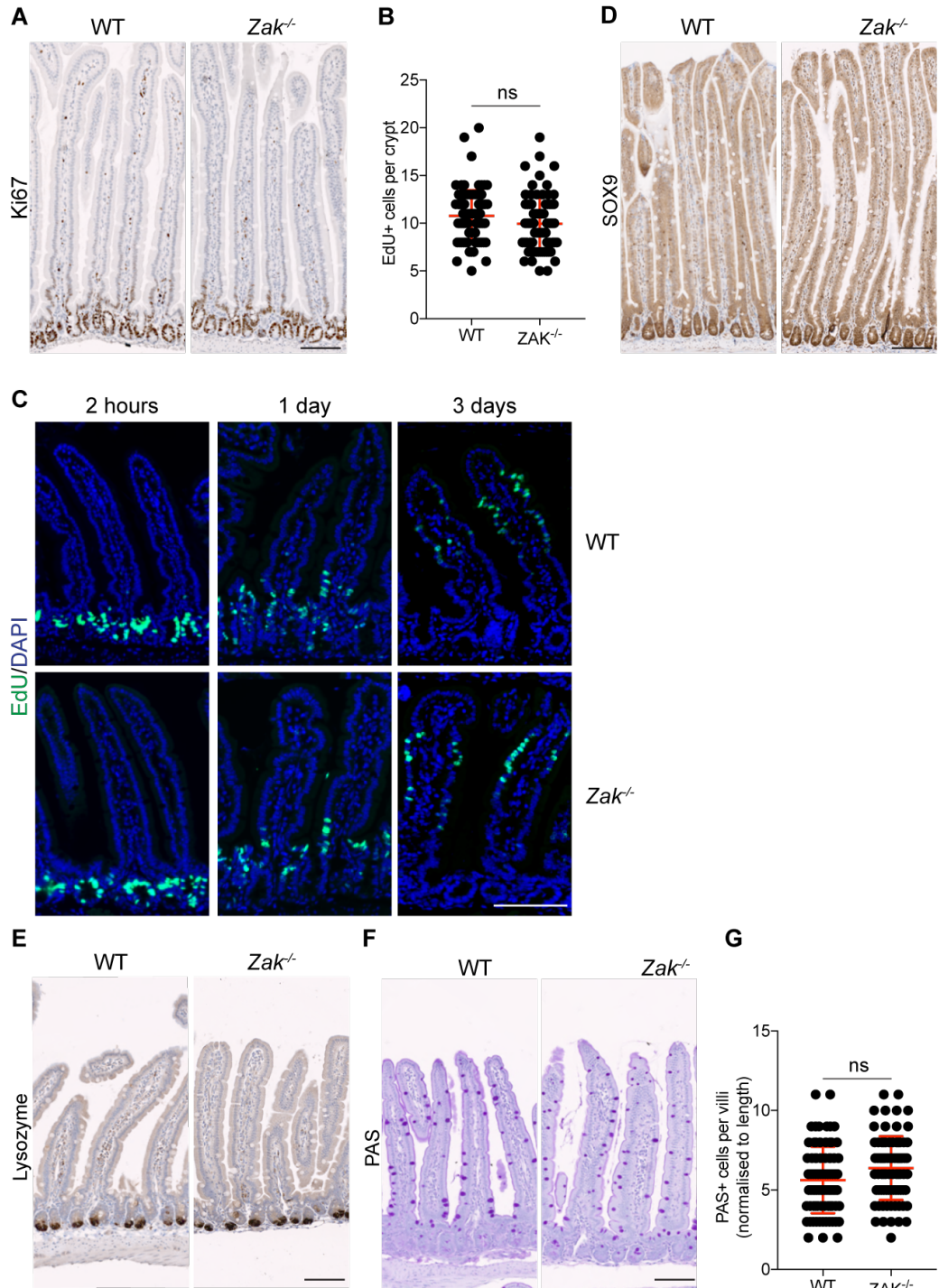


Figure 4.5 *Zak*^{-/-} intestine show normal stem cell proliferation and differentiation.

(A) Representative Ki67 staining in small intestinal sections of WT and *Zak*^{-/-} mice. (B) Quantification of number of EdU⁺ cells per crypt in WT and *Zak*^{-/-} mice 2 hours after EdU administration. (C) Representative immunofluorescent images of EdU tracing between WT and *Zak*^{-/-} intestine. Mice were injected with EdU and collected 2 hours, 1 day and 3 days after injection. (D) Representative staining of the Wnt target- SOX9, Paneth cell marker-Lysozyme (E) and goblet cell marker-PAS (F) in

WT and *Zak*^{-/-} intestines of N=4/group (G) Quantification of number of PAS⁺ cells per villi, normalized to length of the villi in WT and *Zak*^{-/-} mice. To determine the number of EdU⁺ and PAS⁺ cells, 30 crypts or villi were analysed, respectively in three mice/group. Dots represent the counting for the total 90 crypts and villi and means \pm SD of the total 3 mice are shown in red. ns - non-significant ($p>0.05$). Scale bars, 100 μ m.

4.2.4 Loss of *Zak* promotes intestinal regeneration after radiation-induced injury

In response to radiation-induced damage, the intestinal epithelium initiates the regeneration process by enhancing Wnt/ β -catenin signalling to drive cell proliferation (Kabiri et al., 2014). Since our *in vitro* data showed that ZAK is a negative regulator of Wnt, we further investigated the role of ZAK in intestinal regeneration after radiation-induced injury.

Wild-type and *Zak*^{-/-} animals were exposed to a sub-lethal, whole-body dose of irradiation (10 Gy) and the intestinal tissues were collected 3 days later. Enlarged and hyperproliferative crypts were observed in *Zak*^{-/-} intestines 3 days post-irradiation (Figure 4.6A-B). Intestinal crypt length was measured in all intestinal regions for comparison. All regions of the small intestine (duodenum, jejunum, ileum) show increased crypt length in *Zak*^{-/-} mice compared to WT. Colon shows no significant increase in *Zak*^{-/-} mice, which might be explained by the fact that colon is relatively resistant to radiation (Potten and Grant, 1998). Moreover, incomplete recovery, characterised by regions where crypts were absent, was frequently observed in WT animals at this time point, whilst this was almost never observed in *Zak*^{-/-} mice (Figure 4.6A – arrowheads). This indicates that deletion of *Zak* accelerates regeneration of the intestinal epithelium upon irradiation. We confirmed hyperproliferation of the intestinal crypts in the *Zak*-depleted intestine by Ki67 and EdU staining (Figure 4.6C -D). An average of 27 \pm 9 EdU⁺ cells/crypt was observed in *Zak*^{-/-} intestines compared with 15 \pm 6 in WT crypts (Figure 4.6E). We then analysed the expression of the stem cell marker *Olfm4* which was significantly higher in *Zak*^{-/-} intestinal crypts compared to WT (Figure 4.7A). Expression of the Wnt targets SOX9 and CYCLIN D1 were also higher in the *Zak*^{-/-} intestinal crypts, suggesting upregulation of Wnt/ β -catenin signalling in *Zak*-deleted intestine (Figure 4.7B-C). An average of 48 \pm 2 CYCLIND1⁺ cells/crypt was observed in *Zak*^{-/-} intestines compared with 31 \pm 1 in WT crypts. Interestingly, the hyperproliferation phenotype in the *Zak*^{-/-} intestine disappeared 6 days after irradiation when normal intestinal architecture was restored (Figure 4.8A). Similar intestinal proliferation rate and crypt length were observed in both *Zak*^{-/-} and WT mice at this time point (Figure 4.8B-C). The results highlight the unique role of ZAK in regulating intestinal regeneration after irradiation-induced injury when Wnt

signalling is activated to support the stem cell recovery, which is in contrast to its dispensable role during normal homeostasis.

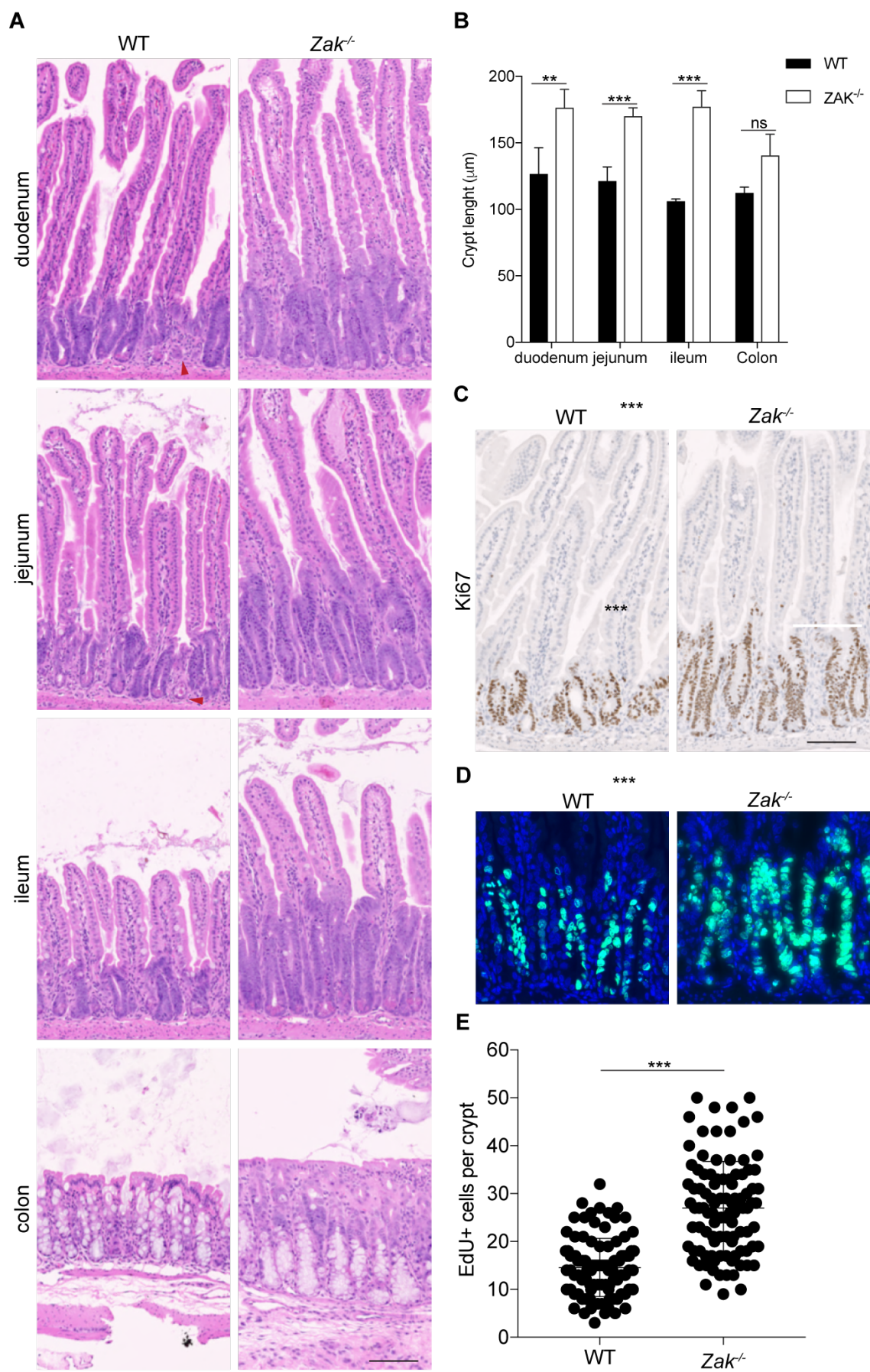


Figure 4.6 Depletion of *Zak* promotes intestinal regeneration after radiation-induced injury.

(A) Representative H&E staining of all 3 regions of small and large intestine in WT and *Zak*^{-/-} mice 3 days after whole-body exposure to 10 Gy radiation. Arrows indicate regions where crypts are absent. (B) Measurement of crypt length in WT and *Zak*^{-/-} animals in all regions of intestine. 30 villi/region were measured and means \pm SD of 3 mice per group are shown. (C) Representative Ki67 staining of the intestine 3 days post-irradiation (dpi) showing increased proliferation in *Zak*^{-/-} mice compared to WT. (D) Representative immunofluorescent staining showing increased number of EdU⁺ cells in *Zak*^{-/-} intestinal crypts compared with WT at 3dpi. (E) Quantitation of number of EdU⁺ cells per crypt in WT and *Zak*^{-/-} mice. Numbers are mean \pm SD of 100 crypts from a representative animal. Scale bars, 100 μ m. ***P \leq 0.001, **P \leq 0.01, ns - non-significant (p > 0.05).

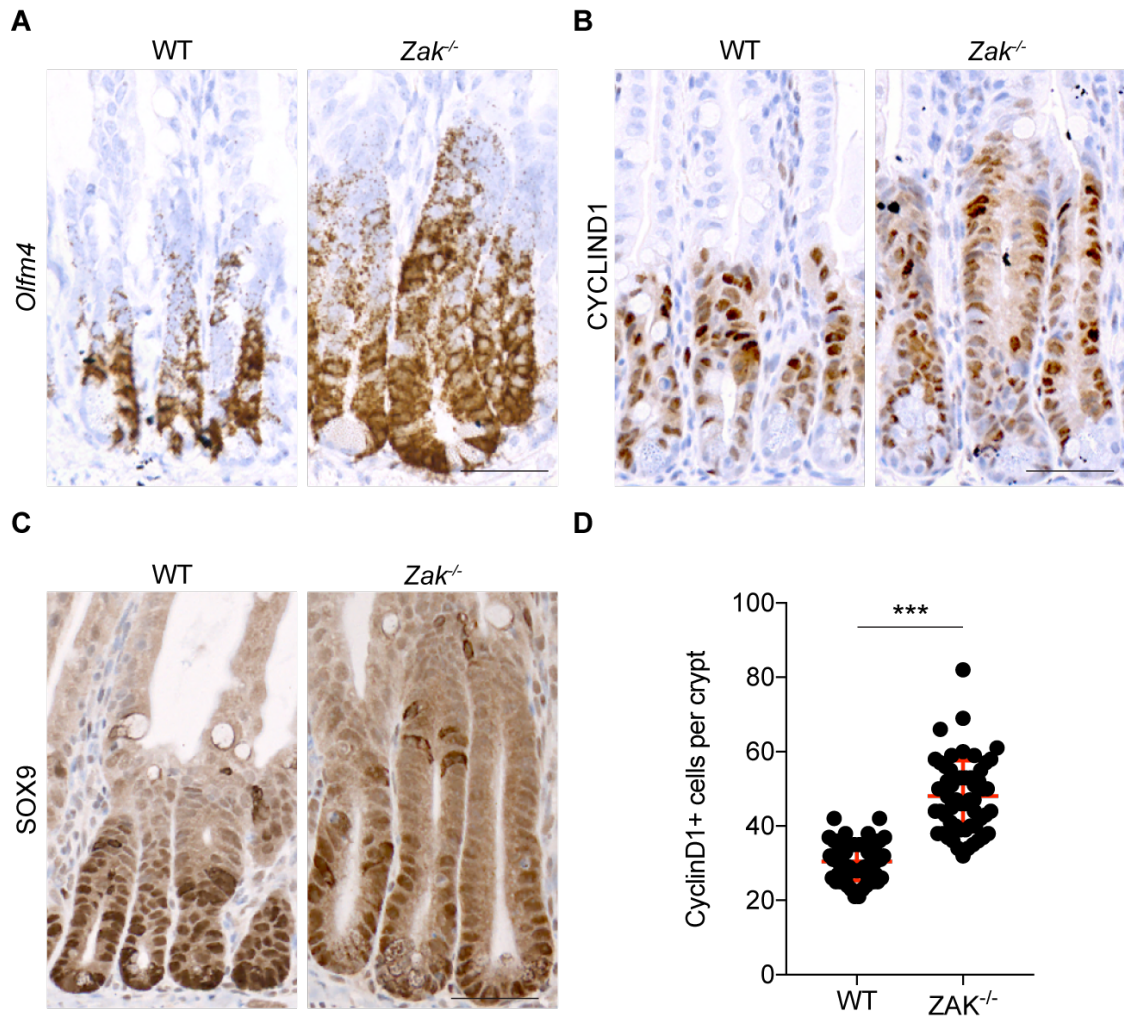


Figure 4.7 Depletion of *Zak* increases expression of ISC markers and Wnt targets after radiation-induced injury.

(A) Representative image of RNAscope ISH of the stem cell marker, *Olfm4*, in intestinal crypts. (B) Representative Cyclin D1 and SOX9 (C) staining showing increases in Wnt/ β -catenin signalling in *Zak*^{-/-} mice compared to WT. N=3/group (D) Quantification of CyclinD1⁺ cell number per crypt. 20 crypts were analysed in two mice per group. Data represents means \pm SD. ***P \leq 0.001. Scale bars, 100 μ m.

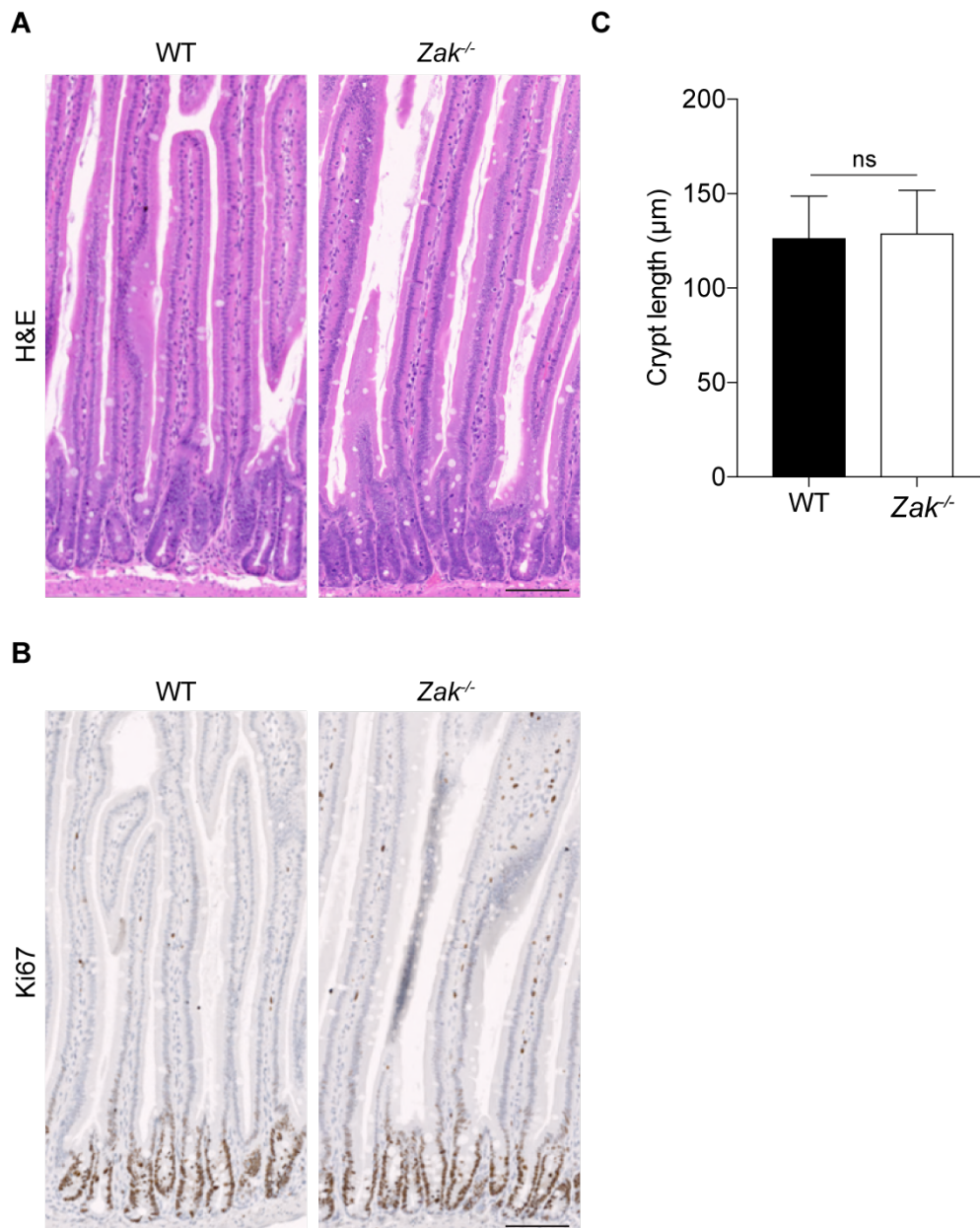


Figure 4.8 *Zak*^{-/-} mice show normal intestinal phenotype 6 days after radiation-induced injury.

(A) Representative H&E staining of intestinal tissues obtained from WT and *Zak*^{-/-} mice 6 days after whole body exposure to 10 Gy radiation. (B) Representative Ki67 staining of intestinal tissues showing no differences in proliferation between *Zak*^{-/-} and WT animals. (C) Measurement of crypt length in WT and in *Zak*^{-/-} mice. 30 crypts/region were measured and means \pm SD of 2 mice are shown. ns - non-significant ($p > 0.05$). Scale bars, 100 μ m.

It has been reported that high Wnt/ β -catenin signalling is required for the *ex vivo* culture of intestinal organoids to sustain their growth (de Lau et al., 2011; Sato et al., 2009). Presence of the potent Wnt agonist, R-spondin, in the culture medium is essential in promoting intestinal stem cell proliferation. I hypothesised that the high Wnt culture conditions used for *ex vivo* organoid culture may resemble, to some extent, intestinal regeneration *in vivo*. To test if *Zak* deletion had any effect on organoid culture, intestinal crypts were isolated from *Zak*^{-/-} and WT intestines and *ex vivo* organoid cultures established. Interestingly, crypts isolated from *Zak*-depleted intestine were nearly 2-fold more likely to form organoids than crypts isolated from WT (Figure 4.9A-B). This could be due to the increase in number of stem cells in the *Zak*-depleted crypts, or the enhanced Wnt/ β -catenin signalling upon *Zak* deletion that potentiates the capacity to form organoids. Altogether, the results suggest that ZAK is essential in regulating intestinal stem cell proliferation when Wnt signalling is activated.

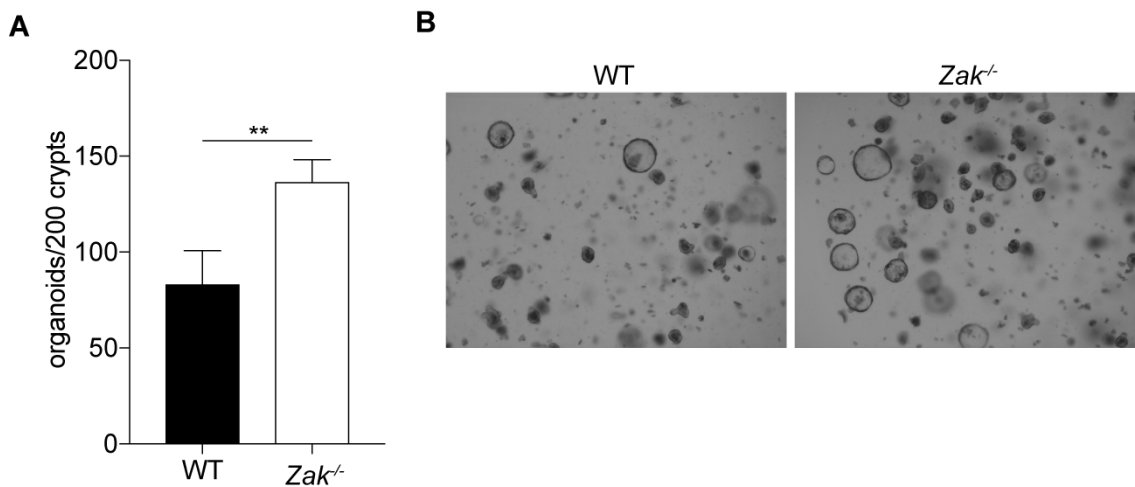


Figure 4.9 Intestinal crypts isolated from *Zak*^{-/-} mice demonstrate higher organoid formation efficiency as compared to WT.

(A) Number of organoids formed per 200 crypts from both WT and *Zak*^{-/-} mice. The number of organoids were counted 3 days after crypt isolation and culture. Data presented are average from four independent experiments (N=4) \pm SD. **P \leq 0.01. (B) Representative images of intestinal organoids derived from *Zak*^{-/-} and WT intestinal crypts after 3 days of culture.

4.3 Discussion

The kinase ZAK has previously been reported to regulate various MAPK pathways, whilst its role in other signalling pathways has yet to be revealed. Our current data show that ZAK inhibits Wnt signalling downstream of β -catenin phosphorylation and ubiquitination in a MAPK-independent mechanism. In the previous chapter, we have shown that ZAK is a Wnt target, expressed exclusively in the intestinal crypts and co-expressed in *Lgr5*-expressing stem cells. Here, we further demonstrate that its depletion promotes intestinal stem cell regeneration after irradiation-induced injury. Taken together, our data suggest that ZAK is a negative regulator of Wnt present at the bottom of the crypts, where it acts to regulate intestinal stem cell proliferation via a negative feedback mechanism in conditions of Wnt/ β -catenin signalling hyperactivation, such as tissue damage or high-Wnt *ex vivo* culture. To our knowledge, this is the first time that ZAK has been directly implicated in regulating intestine regeneration by mediating the Wnt/ β -catenin signalling pathway.

Our current data show that ZAK was able to suppress Wnt signalling activated by APC truncation or expression of the β -catenin S33Y mutant. This suggests that the Wnt inhibitory mechanism is downstream of GSK3-mediated phosphorylation and ubiquitination of β -catenin. Further investigation is needed to dissect the precise role of ZAK in Wnt suppression. Interestingly, a tankyase inhibitor has been shown to inhibit Wnt signalling in APC truncated CRC cells by stabilising the AXIN protein. It would be of interest to test if ZAK also functions to stabilise AXIN. Alternatively, ZAK may act further downstream by regulating nuclear β -catenin/TCF transcription. Although ZAK protein expression was detected exclusively in the cytoplasm, it would still be interesting to explore if ZAK could regulate levels of nuclear β -catenin or TCF proteins.

In this chapter, we have generated *Zak*-null mice for *in vivo* studies. During my research project, Spielman and colleagues reported that complete knockout of both *Zak* isoforms using CRISPR targeting results in early embryonic lethality at E9.5, due to heart malformation and growth retardation (Spielmann et al., 2016). Contrary to their findings, we managed to obtain viable and fertile *Zak*-null animals using the same sgRNA as described in their publication for CRISPR targeting. It is unclear what causes the major discrepancy between our findings and their publication but

there are two differences in the targeting methodology that are worth considering. Firstly, Spielman and colleagues performed gene editing by transfecting mouse embryonic stem cells (ESCs) with the plasmid constructs for CRISPR/Cas9 genome editing system, whilst we directly injected the Cas9 and sgRNA mRNA into one-cell mouse embryos. Secondly, the mice strains used in the two studies are slightly different. In this study, the *Zak-null* mice were generated in both C57BL/B6 and CBAxBL6 background, whilst Spielman used 129xC57BL/6 F1 hybrid ESCs for targeting in their study. In addition, it has been reported that the amounts of Cas9 expression can increase the frequency of off-target effects (Ran et al., 2013). Potential off-target mutations should thus be considered, particularly when the Cas9 plasmid was stably transfected into the ESCs in Spielman's study.

As previously mentioned, ZAK can activate ERK, p38 and JNK pathways (Cho et al., 2004; Liu et al., 2000a; Yang, 2002). Recent studies reveal that MAPK signalling plays an important role in regulating intestinal stem cell-fate decision (Basak et al., 2016; Gupta et al., 2014; Heuberger et al., 2014) and tumorigenesis (Abaco et al., 1996; Janssen et al., 2006; Jeong et al., 2012). In particular, activation of MAPK promotes goblet cell over Paneth cell differentiation, whilst enteroendocrine cell differentiation requires inhibition of MAPK signalling. However, no significant differences were observed in the number of Paneth cells or goblet cells between WT and *Zak*-null animals. This could be due to the redundancy of other MAP3Ks in the intestine. Alternatively, the role of ZAK in regulating both MAPK and Wnt signalling may represent a unique functional role in the intestine different from other MAPK family members. In addition, we are currently performing Chromogranin A immunohistochemistry to further investigate any possible effect on enteroendocrine cell differentiation in the *Zak*^{-/-} mice. Our preliminary data suggest that the Wnt inhibitory role of ZAK is MAPK-independent. However, the potential crosstalk between MAPK and Wnt signalling cannot be completely excluded. Although the MAPK inhibitors have been widely used in other studies, the efficiency of the inhibitors should be confirmed in our system by examining changes in phosphorylation of MAPKs. Moreover, the compensatory roles of different MAPKs such as ERK5 and ERK1/2 has been previously described (de Jong et al., 2016). Therefore, treatments of various combined MAPK inhibitors should be examined for comprehensive analysis of the Wnt regulatory role of ZAK.

One interesting finding in this project is that ZAK appears to be required to regulate intestinal regeneration after damage but is dispensable during tissue homeostasis. These results provide compelling evidence for the hypothesis that ZAK contributes to the negative feedback loop at the crypt bottom to fine tune Wnt/ β -catenin signalling when it is hyperactivated. It is possible that ZAK plays a role in the intestine predominantly during hyperproliferative conditions such as tumorigenesis, regeneration, inflammatory damage or *ex vivo* organoid culture. It would be interesting to investigate other pathological models to better understand the functional significance of ZAK in intestine in such scenarios. Moreover, as mentioned in section 4.2.4, WT animals frequently show regions where crypts were absent 3 days after irradiation, whilst this was almost never observed in *Zak*^{-/-} mice. This raises the question whether deletion of *Zak* accelerates regeneration of the intestinal epithelium upon irradiation or *Zak*-null animals are more resistant to damage from the start. To answer this question, we will analyse mice at short time points after irradiation, particularly 6 hours and 1 day after radiation.

ZAK was initially identified in the Stem-Cell/Wnt cluster from the TCGA expression data, where changes in ZAK expression were observed between the different subtypes of CRCs (see Chapter 3). It would be important to further study the role of ZAK in intestinal tumour initiation and progression. We are currently generating *Zak*^{-/-} *Apc*^{min} mice to further characterise the effect of *Zak* deletion in intestinal tumourigenesis.

In summary, our data show that ZAK negatively regulates intestinal stem cell proliferation by inhibiting the Wnt signalling pathway. This is in contrary to the previous reports proposing that ZAK is an oncogene (Cho et al., 2004; Liu et al., 2014b; Rey et al., 2015). This is mainly due to the observation that ZAK is generally upregulated in various cancers, but functional characterisation in those studies were limited to simple *in vitro* assays in established cancer cell lines. However, these observations described in this chapter should be interpreted with caution. As demonstrated in the previous chapter, ZAK is potentially a Wnt target gene. It is therefore not surprising that ZAK, and many other Wnt targets such as *AXIN2* and *RNF43*, are upregulated in *APC*-mutated CRCs. ZAK is mostly associated with the MAPK pathway in the current literature, whilst its role in Wnt signalling regulation is largely unknown. Importantly, the functional role of ZAK in the intestine has never been examined *in vivo*. Our current data provide the first *in vivo* evidence that ZAK

Chapter 4. The ZAK kinase is a Wnt negative regulator expressed by stem cells

is involved in repressing stem cell proliferation during intestinal regeneration by negatively regulating Wnt signalling. Further studies of the *Zak*^{-/-} mice in intestinal tumour models will help to re-evaluate the role of ZAK in CRC.

Chapter 5. SH3BP4 inhibits intestinal tumour initiation and growth by negative regulation of Wnt signalling

5.1 Introduction

SRC homology 3 domain-binding protein 4 (SH3BP4) encodes a 963 amino acid protein with multiple structural domains and sequence motifs. The protein consists of two SH3 domains, one at the N-terminus (SH3_1 family member) and a second one close to the C-terminus (SH3_2 family member). The SH3 domain is a well characterised family of protein-interaction modules (Mayer, 2001) (Figure 5.1A). Through selective binding affinity to proline-rich ligands, this domain plays an important role in many biological processes such as enzyme regulation by modulating intramolecular interactions, modulating the subcellular localisation of components of various signalling pathways and mediating large multiprotein complex assembly (Mayer, 2001). The N-terminal region of SH3BP4 also contains three conserved motifs: LIDL motif (a putative clathrin-binding motif), AP2 α (an adaptor protein 2 α) and three NPF repeats (Asparagine-Proline-Phenylalanine - putative Eps15 homology (EH) domain-binding sites) (Kokoszynska et al.). The amino acids 317 to 454 comprise the protein-protein interaction ZU5 domain. The C-terminus contains two death domain (DD) family members, which mediate self-association with DDs of other proteins to induce pro-apoptotic signals (Kokoszynska et al.). A ZU5-DD architecture has also been found in proteins related to extracellular signal transduction such as the netrins (Reed et al., 2004; Wang et al., 2009) and in the scaffold proteins, ankyrins (Ipsaro et al., 2009).

Another recently identified protein, metastasis-associated in colon cancer protein 1 (MACC1), shares approximately 45% homology with SH3BP4 in both human and mouse. Expression of MACC1 has previously been associated with poor prognosis in CRC (Stein et al., 2009). These similarities are evolutionarily conserved in other vertebrates, such as fish and birds (Stein et al., 2010). The two proteins

contain similar domains and sequence motifs, apart from that the N-terminal SH3 domain is absent in MACC1.

SH3BP4 has previously been shown to play a regulatory role in multiple signalling pathways. Tosoni and colleagues first described the functional role of SH3BP4 in regulating clathrin-mediated internalisation of the transferrin receptor (TfR) (Tosoni et al., 2005). This effect was shown to be dependent on the SH3_1 domain that binds to the GTPase dynamin in the clathrin-coated vesicle (Tosoni et al., 2005). In addition, SH3BP4 has also been demonstrated to regulate fibroblast growth factor receptor (FGFR) trafficking and the subsequent cellular responses (Francavilla et al., 2013). In another study, Kim et al. reported SH3BP4 as a negative regulator of Amino Acid-Rag GTPase-mTORC1 signalling (Kim et al., 2012b). Amino acids stimulate mTOR signalling via Rag GTPases, such as GTP-bound RagA or RagB. SH3BP4 preferentially binds to inactive GDP-bound Rag GTPase via the SH3 domain and inhibits the formation of the active Rag GTPase complex, thus resulting in inactivation of mTOR signalling (Kim et al., 2012b).

The possibility of the regulation of *SH3BP4* gene expression by microRNAs (miRNAs) has been observed in three independent studies. Microarray analysis revealed enrichment of *SH3BP4* expression in metastatic prostate cancer (Zhang et al., 2016). Then, a subsequent bioinformatics analysis of miRNAs networks further suggested that *SH3BP4* expression could be modulated by miR-182 (Zhang et al., 2016). More recently, SH3BP4 has also been reported as a melanogenesis-related gene targeted by miR-125b (Kim et al., 2017). The study showed that *SH3BP4* expression is decreased through direct binding of miR-125b to the *SH3BP4* 3'-UTR, which results in reduced number of melanocytes.

In Chapter 3, we demonstrated that *SH3BP4* is differentially expressed in human CRCs and that its expression is restricted to the crypt compartment in normal intestine. However, the role of SH3BP4 in the intestine has never been studied. Here we examine the putative role of SH3BP4 in Wnt/ β -catenin signalling and study its functional significance in intestinal homeostasis and tumorigenesis *in vivo*.

5.2 Results

5.2.1 SH3BP4 is a negative regulator of Wnt/ β -catenin signalling

We have previously confirmed that *Sh3bp4* is expressed in the ISC compartment. Expression of *Sh3bp4* is further enriched in the *Apc*-mutated intestinal tumours and organoids, suggesting that *Sh3bp4* is transcriptionally regulated by Wnt/ β -catenin signalling. To gain insight into the role of SH3BP4 in Wnt/ β -catenin signalling, I first deleted SH3BP4 in HEK293T cells using the CRISPR/Cas9 gene editing system. A sgRNA targeting the first exon of *SH3BP4* was designed to target *SH3BP4* gene. Homozygous frameshift mutations in the selected CRISPR-targeted HEK293T clone (Δ SH3BP4) was confirmed by sequencing (Figure 5.1B). A 7bp- and a 5bp deletion were retrieved from the Δ SH3BP4 clone, resulting in protein truncation at 110 and 89 amino acids respectively. Protein depletion of SH3BP4 in the Δ SH3BP4 cells was confirmed by Western blot analysis (Figure 5.1C). Deletion of SH3BP4 resulted in ~2.5-fold increase in Wnt3A-induced TCF-TopFlash reporter transcriptional activity (Figure 5.1D) and an increase in active β -catenin protein levels (Figure 5.1E). To confirm the Wnt signal activation in Δ SH3BP4 cells, mRNA expression of the Wnt target genes AXIN2, CCND1 and MYC was further analysed by quantitative RT-PCR (Figure 5.1F). Consistently, significant upregulated expression of the Wnt target genes was detected in Δ SH3BP4 cells when compared with wild-type HEK293T cells.

In a reverse experiment, SH3BP4 was overexpressed in HEK293T cells. In accordance with the Δ SH3BP4 data, expression of wild-type SH3BP4 significantly suppressed Wnt3A-induced TCF-TopFLASH reporter activity (Figure 5.2A). The Wnt signal inhibition was confirmed by reduced active β -catenin protein levels (Figure 5.2B). As mentioned above, MACC1 shares high homology with SH3BP4 in terms of domains and sequence motifs. I therefore tested if MACC1 could play a similar role in Wnt/ β -catenin signalling. However, overexpression of MACC1 did not show any inhibition of Wnt3A-induced TCF-TopFLASH reporter activity (Figure 5.2C), suggesting that MACC1 does not modulate Wnt signalling despite sequence homology with SH3BP4. Taken together, these data support the notion that SH3BP4 is a negative regulator of Wnt/ β -catenin signalling.

Chapter 5. SH3BP4 inhibits intestinal tumour initiation and growth by negative regulation of Wnt signalling

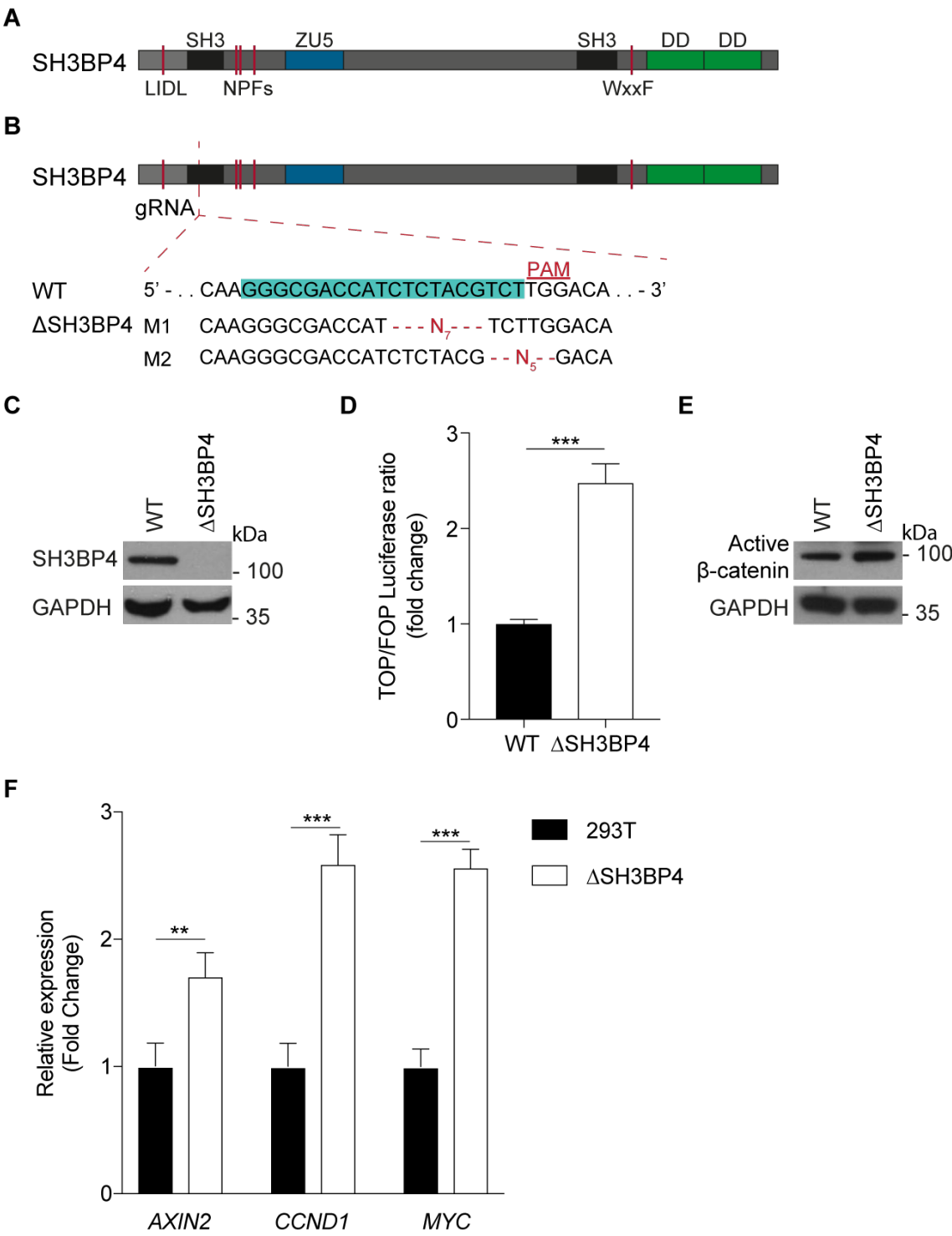


Figure 5.1 Deletion of SH3BP4 activates Wnt/ β -catenin signalling.

(A) SH3BP4 protein domains and motifs. (B) Schematic representation of the CRISPR/Cas9 targeting strategy the first exon of *Sh3bp4*. Genotyping of the selected Δ SH3BP4 mutant clone reveals homozygous frameshift mutations on both alleles, indicated as M1 and M2. (C) Immunoblot showing protein loss in the Δ SH3BP4 mutant clone. (D) Relative Wnt3a-induced TCF-TopFlash reporter activity in HEK293T wild-type and Δ SH3BP4 cells. The Top and Fop signals were normalized to the renilla luciferase signal and averages \pm SD of four independent experiments are shown (E) Western blot analysis of active (non-phosphorylated) form of β -catenin. (F) Activation of the Wnt target genes *AXIN2*, *CCND1* and *MYC* was examined in HEK293T cells by quantitative RT-PCR. Expression data are presented as fold induction normalised to GAPDH. Data represent means of three independent experiments \pm SD. **P<0.01, ***P< 0.001.

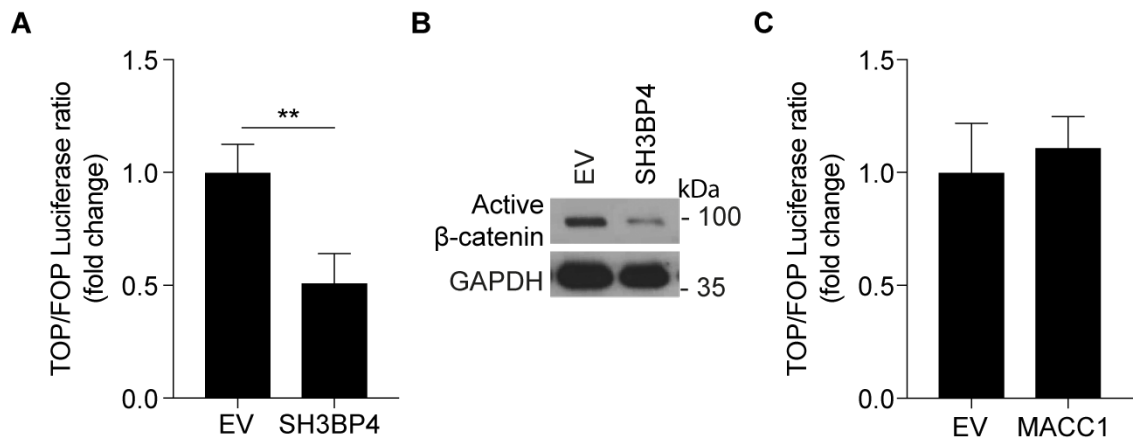


Figure 5.2 SH3BP4, but not MACC1, inhibits Wnt/β-catenin signalling.

(A) Expression of SH3BP4 in HEK293T cells inhibits Wnt3A-induced TCF-TopFlash reporter activity. (B) Overexpression of SH3BP4 significantly reduces active β-catenin level as revealed by Western blot analysis. (C) Relative TCF-TopFlash reporter activity in Wnt3A-induced HEK293T cells expressing empty-vector or MACC1. Averages of four independent experiments \pm SD are shown. **P<0.01.

5.2.2 SH3BP4 inhibits Wnt signalling downstream of β -catenin phosphorylation/ubiquitination

To understand the mechanism by which SH3BP4 inhibits Wnt/ β -catenin signalling, we followed a similar approach to that previously described in Chapter 4 for ZAK, and activated the Wnt pathway at different subcellular levels together with SH3BP4 expression. Expression of SH3BP4 was able to inhibit TCF-TopFLASH reporter activity induced by the constitutively active form of LRP6 (Δ N-LRP6) (Figure 5.3A), suggesting that SH3BP4 acts downstream of LRP6 phosphorylation. Next, we tested if SH3BP4 can inhibit Wnt signalling induced by GSK3 β inhibitor (CHIR99021). CHIR99021 inhibits GSK3 β -mediated β -catenin phosphorylation at Thr41, Ser37 and Ser33 within the destruction complex, which consequently stabilises β -catenin. TCF-TopFLASH luciferase assay showed that SH3BP4 significantly inhibited CHIR99021-induced Wnt/ β -catenin signalling (Figure 5.3B and D). This result was further confirmed by expressing the constitutively active form of β -catenin (β CatS33Y) to activate Wnt signalling. Once again, SH3BP4 was able to inhibit Wnt signalling induced by the phospho-mutant β CatS33Y (Figure 5.3C and D). These data indicate that SH3BP4 inhibits Wnt signalling downstream of β -catenin phosphorylation.

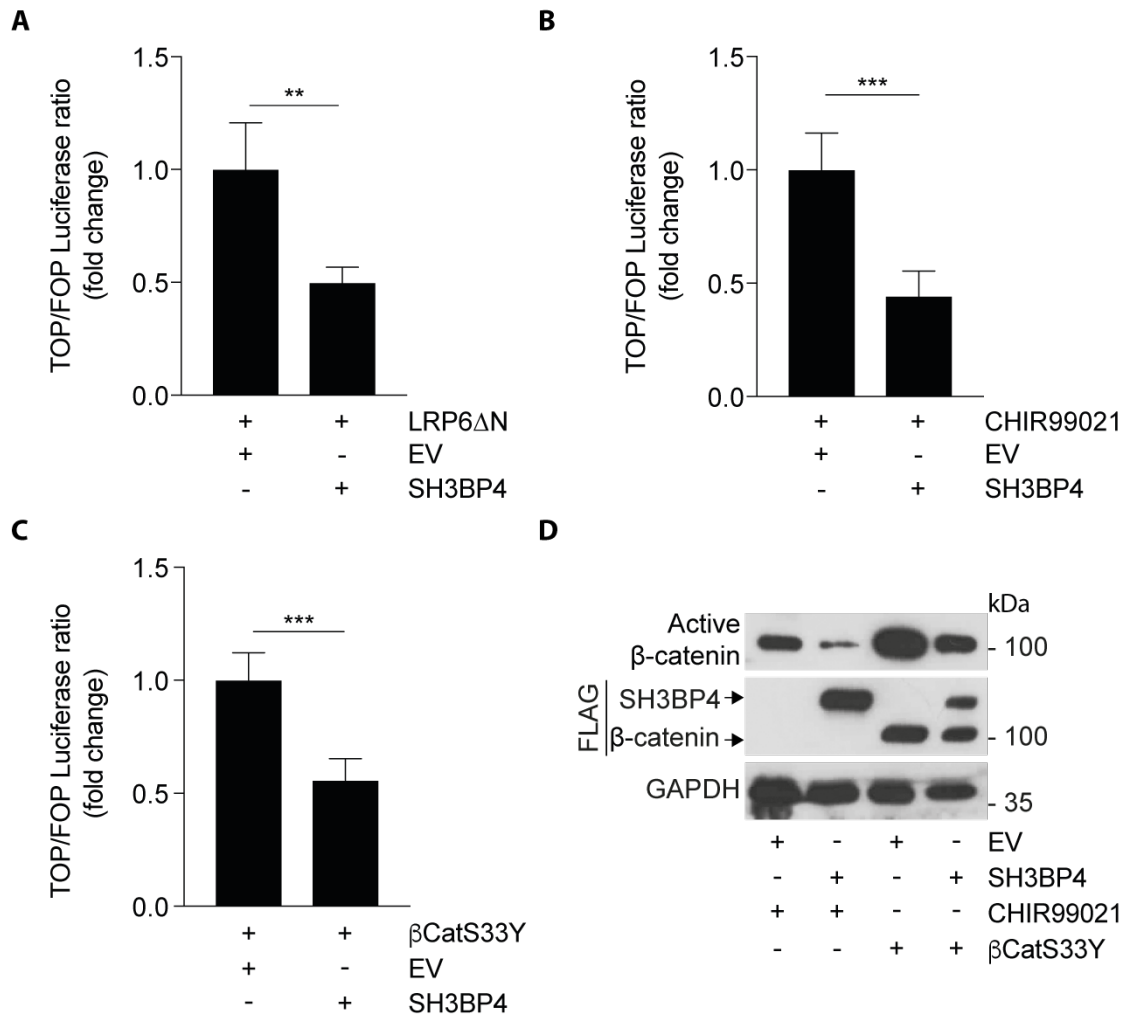


Figure 5.3 SH3BP4 inhibits Wnt signalling downstream of β -catenin phosphorylation.

(A) Expression of SH3BP4 in HEK293T cells significantly inhibits Δ N-LRP6-induced TCF-TopFlash reporter activity. (B) HEK293T cells overexpressing EV and SH3BP4 were treated with 2 μ M of GSK3 inhibitor CHIR99021 for 24 hours and relative TCF-TopFlash reporter activity was measured. (C) SH3BP4 suppresses TCF-TopFlash reporter activity induced by a stabilised form of β -catenin (β -CatS33Y). Averages of four independent experiments \pm SD are shown. ***P<0.001, **P<0.01. (D) Western blot shows that SH3BP4 reduces the levels of active β -catenin induced by GSK3 inhibition and β -cateninS33Y expression.

Next, we attempted to identify the specific domain or motif of SH3BP4 that is responsible for Wnt signal inhibition. Five different SH3BP4 deletion/mutant constructs lacking various SH3BP4 domains were generated (Figure 5.4A), where similar protein expression levels were observed in all constructs (Figure 5.4B). A construct lacking the clathrin-binding LIDL motif at the N terminus (SH3BP4^{ΔN}) was generated due to its reported role in receptor trafficking (Francavilla et al., 2013; Tosoni et al., 2005) and clathrin-mediated LRP6 receptor endocytosis (Liu et al., 2014a). For the first SH3 domain (SH3_1), a point mutation was generated to replace the Trp92 with alanine (SH3BP4^{W92A}) – an amino acid that is reportedly conserved and is critical for SH3 domain interactions (Erpel et al., 1995; Tosoni et al., 2005). To test which domain is crucial for Wnt inhibition, we expressed the various SH3BP4 mutant constructs in cells depleted of endogenous SH3BP4 (ΔSH3BP4) and measured the Wnt3A-induced TCF-TopFLASH reporter activity. As expected, expression of WT SH3BP4 readily rescued the Wnt activation caused by endogenous SH3BP4 depletion (Figure 5.4C). Similar to the WT protein, most of the SH3BP4 deletion/mutation constructs were also able to repress Wnt activation. However, one construct lacking the ZU5 domain (SH3BP4^{ΔZU5}) failed to exert full Wnt inhibitory function, suggesting that the inhibitory role of SH3BP4 is dependent on the ZU5 domain (Figure 5.4C).

The Wnt inhibitory role of SH3BP4 was further examined in another *APC* mutant system (HEK293T ΔAPC) described earlier in Chapter 4 (section 4.3), which activates Wnt signalling via suppression of β-catenin ubiquitination. Interestingly, expression of WT SH3BP4, but not SH3BP4^{ΔZU5}, suppressed Wnt activation induced by *APC* truncating mutation (Figure 5.4D). Taken together, the data suggest that SH3BP4 inhibits Wnt signalling downstream of β-catenin phosphorylation and ubiquitination, and is dependent on the ZU5 domain.

We demonstrated earlier that the homologue MACC1 did not inhibit Wnt3A-induced TCF transcription (Figure 5.2C). Here we further tested the potential Wnt inhibitory role of MACC1 using the ΔAPC model. Consistently with our previous data, MACC1 was not able to inhibit Wnt/β-catenin signalling in HEK293TΔAPC either (Figure 5.4E). This consolidates the hypothesis that SH3BP4, but not its homologue MACC1, functions as a negative Wnt regulator.

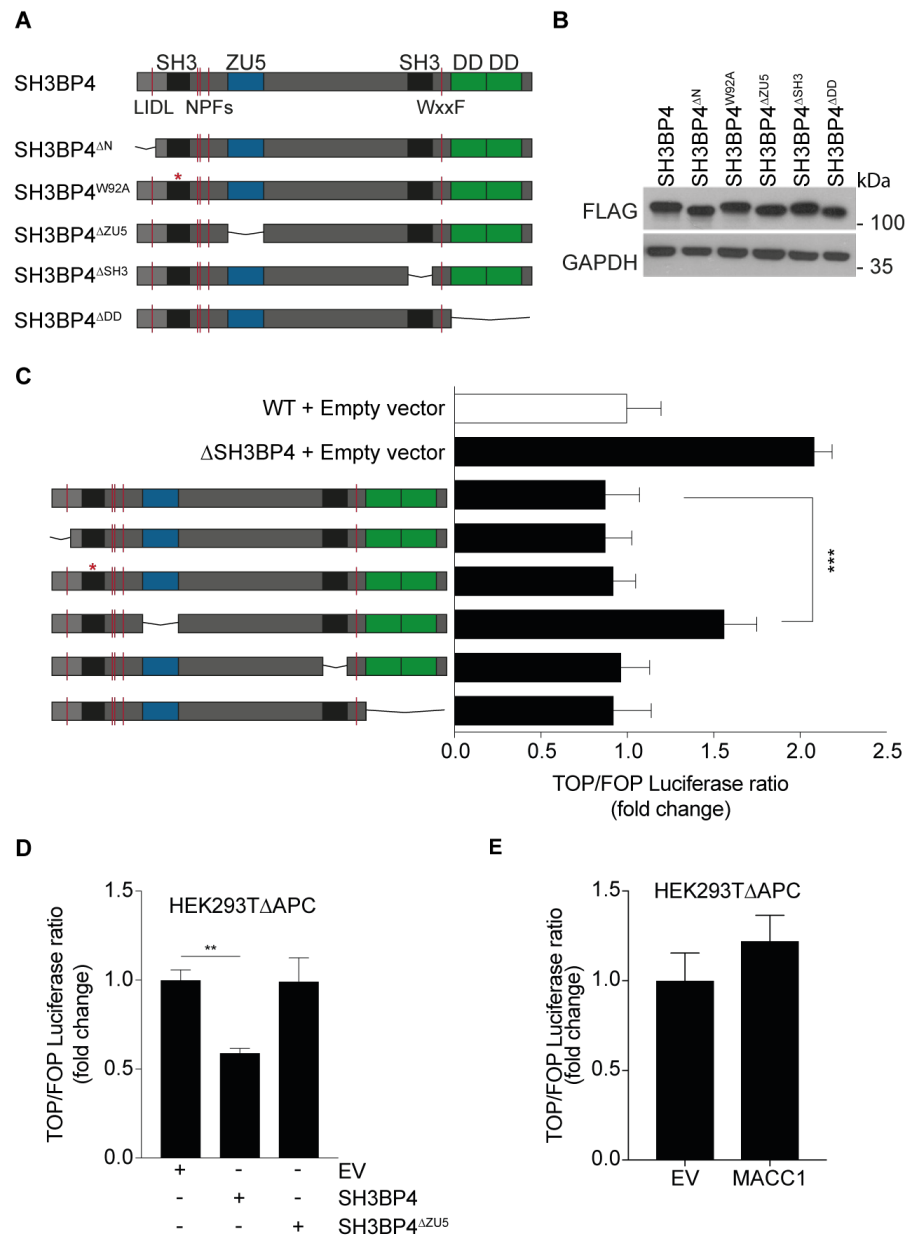


Figure 5.4 SH3BP4-mediated inhibition of Wnt/β-catenin signalling is dependent on the ZU5 domain.

(A) Different FLAG-tagged deletion or mutation constructs of SH3BP4 without specific domains or motifs. (B) Western blot analysis showing similar expression among the different FLAG-tagged constructs of SH3BP4. (C) Relative TCF-TopFlash reporter activity showing that deletion of the ZU5 domain fails to restore the normal levels of Wnt/β-catenin signalling in ΔSH3BP4 cells. (D) Relative TCF-TopFlash reporter activity showing that expression of WT SH3BP4, but not SH3BP4^{ΔZU5}, suppresses Wnt activation induced by APC truncating mutation (ΔAPC). (E) Expression of the homologue MACC1 does not inhibit Wnt activation in ΔAPC cells. Data represent mean of at three independent experiments ± SD. **P<0.01, ***P<0.001.

As described earlier, SH3BP4 has previously been reported to inhibit the mTOR1- pathway (Kim et al., 2012b). Indeed, upregulation of p70S6 phosphorylation was observed in the Δ SH3BP4 cells, confirming the negative regulatory role of SH3BP4 in mTOR signalling (Figure 5.5A). To determine whether the Wnt inhibitory role of SH3BP4 is dependent on mTOR, we examined the Wnt activating effect of the Δ SH3BP4 cells in the presence of the mTOR inhibitor, rapamycin. As expected, rapamycin treatment significantly suppressed mTOR signalling induced by SH3BP4 depletion as indicated by p70S6 phosphorylation (Figure 5.5B). However, suppression of mTOR signalling did not affect Δ SH3BP4-induced Wnt activation, suggesting that the Wnt negative regulatory role of SH3BP4 is independent of mTOR activation (Figure 5.5C). Surprisingly, we saw a further increase in Wnt activation upon rapamycin treatment.

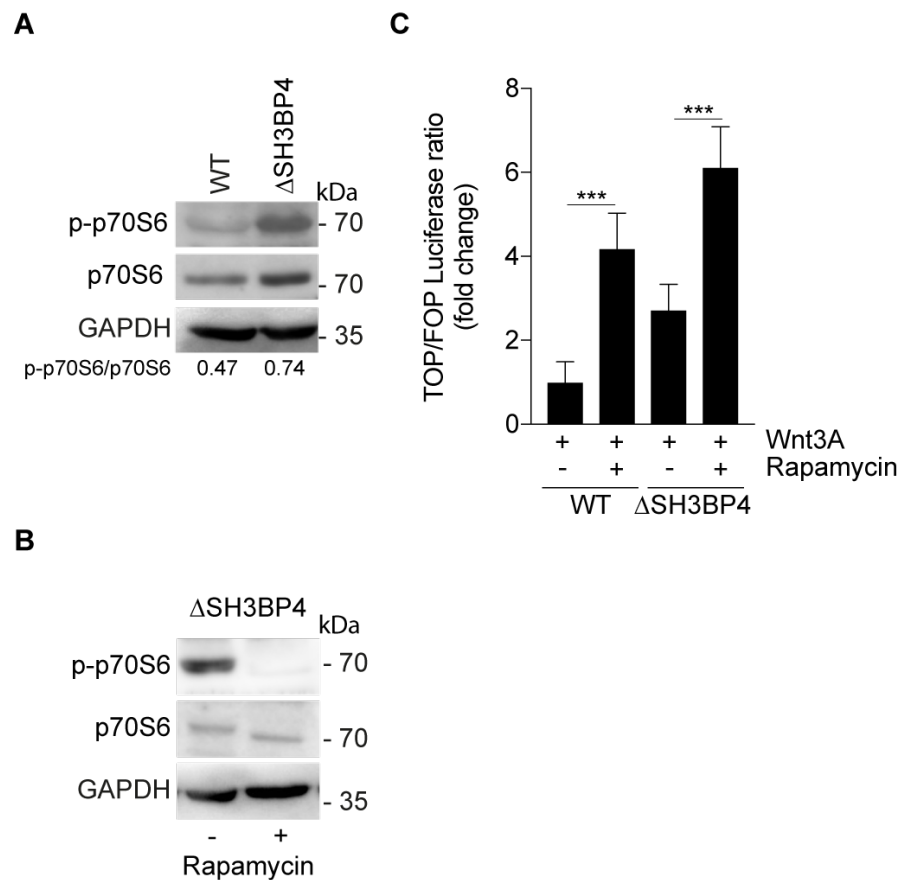


Figure 5.5 Activation of Wnt/β-catenin signalling in ΔSH3BP4 is independent of mTOR pathway activation.

(A) Western blot showing that cells depleted of SH3BP4 have higher levels of phosphorylation of the mitogen activated Ser/Thr protein kinase p70S6 (p-p70S6), confirming that depletion of SH3BP4 activates mTOR pathways as previously described (Kim et al., 2012b). (B) Western blot analysis confirms inhibition of mTOR signalling (p-p70S6) upon rapamycin treatment. (C) HEK293T and ΔSH3BP4 cells were treated with the mTOR inhibitor rapamycin (20nM for 24hours) as shown (non-treated (-); treated (+)) followed by TCF-TopFlash reporter activity measurement. Data represent mean of at three independent experiments ± SD. ***P< 0.001

5.2.3 SH3BP4 interacts with APC and β -catenin

Expression of SH3BP4 has previously been reported to be localised to the plasma membrane, perinuclear region and clathrin-coated vesicles (Kim and Kim, 2013; Kim et al., 2012b; Tosoni et al., 2005). To examine the cellular localisation of the protein, we performed immunofluorescent staining of the endogenous SH3BP4 protein in WT HEK293T and colon cancer cells (SW480 and LS174T). Consistent with the previous studies, the SH3BP4 protein was indeed localised to the nuclei, plasma membrane and vesicles (Figure 5.6A-B). The perinuclear staining was not obvious in HEK293T cells, but was clear in colon cancer cells such as SW480 and LS174T (Figure 5.6B). Our current data show that SH3BP4 inhibits Wnt signalling downstream of β -catenin phosphorylation/ubiquitination, and is strongly localised to the perinuclear membrane and nucleus. This prompted us to speculate that SH3BP4 might regulate β -catenin/TCF4 transcription or nuclear shuttling of β -catenin. Since APC has been reported to regulate β -catenin nuclear transport (Henderson, 2000), we therefore investigated whether SH3BP4 could interact directly with β -catenin, TCF4 and/or APC proteins. To avoid non-physiological overexpression of the protein, LS174T cells (with higher levels of SH3BP4 protein and wild-type APC) were chosen for the subsequent endogenous immunoprecipitation (IP) experiments. In addition, we generated the CRISPR-mediated SH3BP4 deletion (Δ SH3BP4) in LS174T cells using the same sgRNA as described in Figure 5.1B to serve as a negative control for the interaction experiments. TCF4 did not co-immunoprecipitate with SH3BP4. β -catenin interaction was inconclusive as the binding was also faintly detected in the Δ SH3BP4 negative control cells, suggesting non-specificity of the SH3BP4 antibody. APC protein was difficult to detect and not results were inconsistent between experiments (Figure 5.7A). We then performed the reverse co-IP experiments to validate the bindings. Interestingly, SH3BP4 was detected in the β -catenin and APC co-IP complexes (Figure 5.7B-C). It remains unclear whether the binding of SH3BP4 with β -catenin and APC is direct or indirect. Further studies will be needed to investigate whether SH3BP4 can potentially regulate binding between APC and β -catenin, mediating β -catenin nuclear shuttling for example.

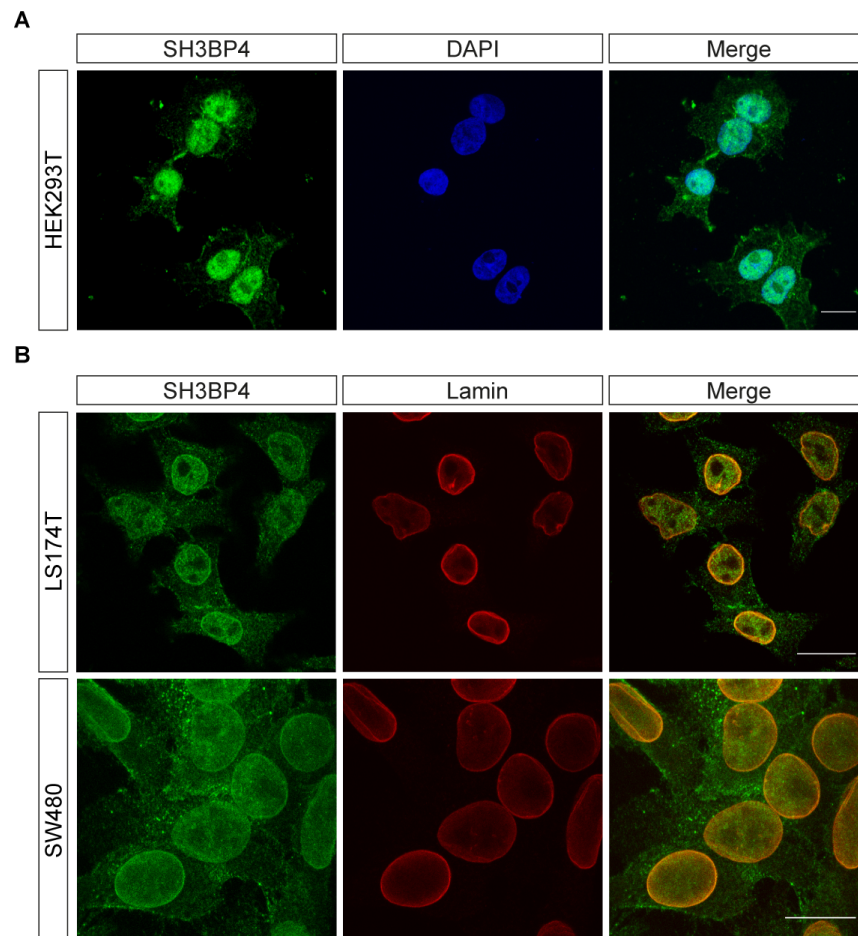


Figure 5.6 SH3BP4 is predominantly localised at perinuclear membrane and nucleus.

(A) Immunofluorescent staining of SH3BP4 in HEK293T cells showing high levels of nuclear protein (B) Z-stack of co-localisation of SH3BP4 and the nuclear membrane marker Lamin in both LS174T (upper panel) and SW480 (lower panel) cells. Scale bars, 100µm.

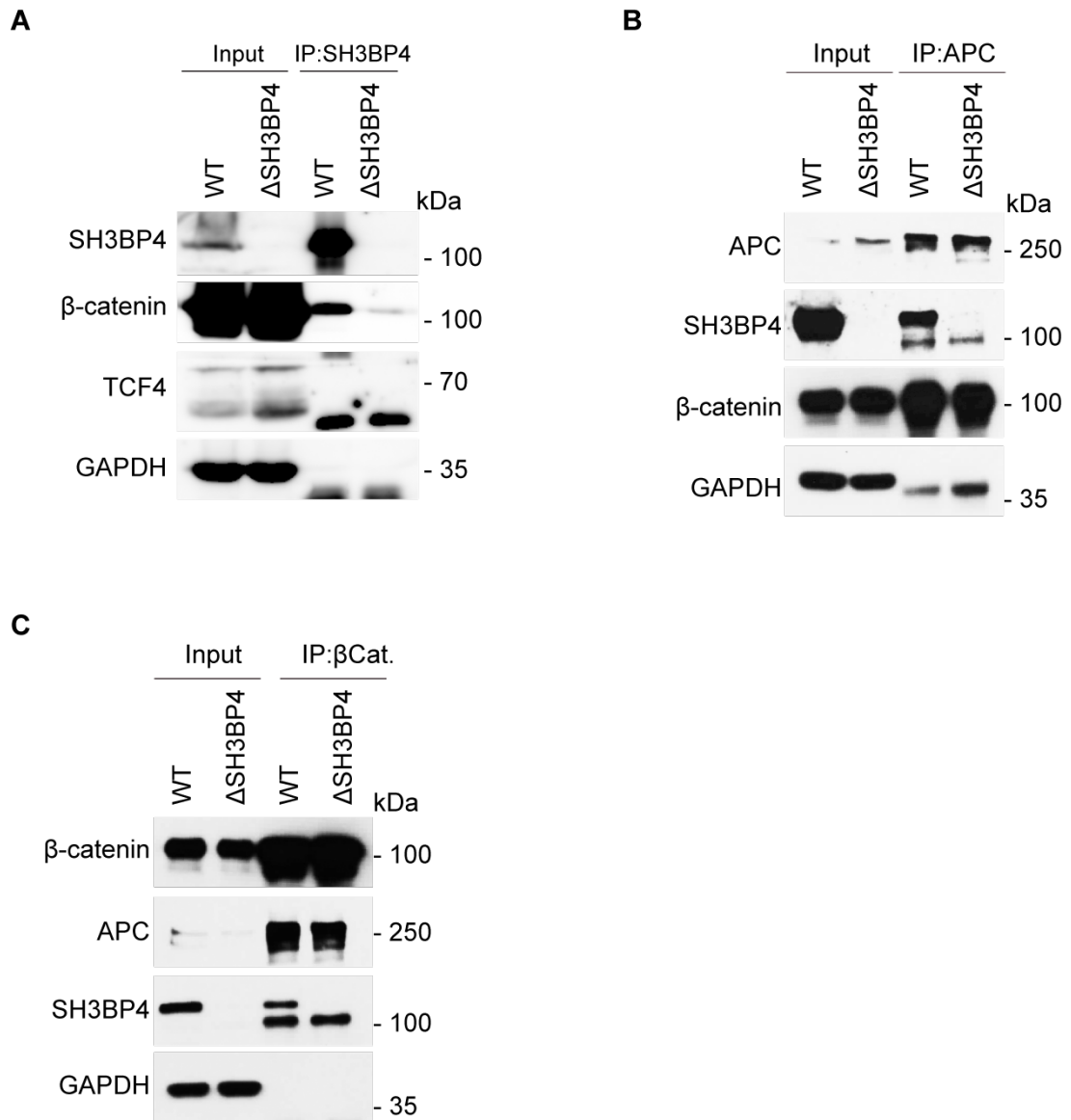


Figure 5.7 SH3BP4 interacts with APC and β -catenin.

LS174T cells were used to immunoprecipitate SH3BP4 (A) APC (B) and β -catenin (C). Δ SH3BP4 LS174T cells were used as negative control. Cell lysates were subjected to SDS-PAGE followed by western blot analysis using the indicated antibodies.

5.2.4 Loss of SH3BP4 promotes intestinal stem cell proliferation

To further understand the biological relevance of SH3BP4 in Wnt/ β -catenin signalling and intestinal homoeostasis, we obtained the conditional knockout mouse model of *Sh3bp4* from the International Mouse Strain Resource generated by the Wellcome Trust Sanger institute (*Sh3bp4*^{tm1a(EUCOMM)Wtsi}). Briefly, two loxP sites were inserted flanking the critical exon 4 that encodes most of the gene. A lacZ sequence followed by neomycin selection cassette under the control of the human β -actin promoter and SV40 polyA was flanked by two FRT (flippase recognition target) sites and was placed upstream of the first loxP site (Figure 5.8A). The presence of the lacZ-neomycin cassette disrupts normal gene transcription and is considered as a null allele. We were not able to obtain homozygous *Sh3bp4*^{tm1a(EUCOMM)Wtsi} pups, indicating that *Sh3bp4* full knock-out is lethal. The mice were then crossed with Tg(CAG-Flpo)1Afst strain to remove the lacZ-neomycin cassette, resulting in the conditional *Sh3bp4*^{fl/fl} mice. Next, *Sh3bp4*^{fl/fl} mice were crossed with the intestinal epithelial-specific Villin^{CreERT2} mice to obtain the final conditional *Sh3bp4*^{fl/fl} Villin^{CreERT2} strain, in which the Cre-mediated *Sh3bp4* deletion could be induced upon intraperitoneal tamoxifen administration (Figure 5.8B).

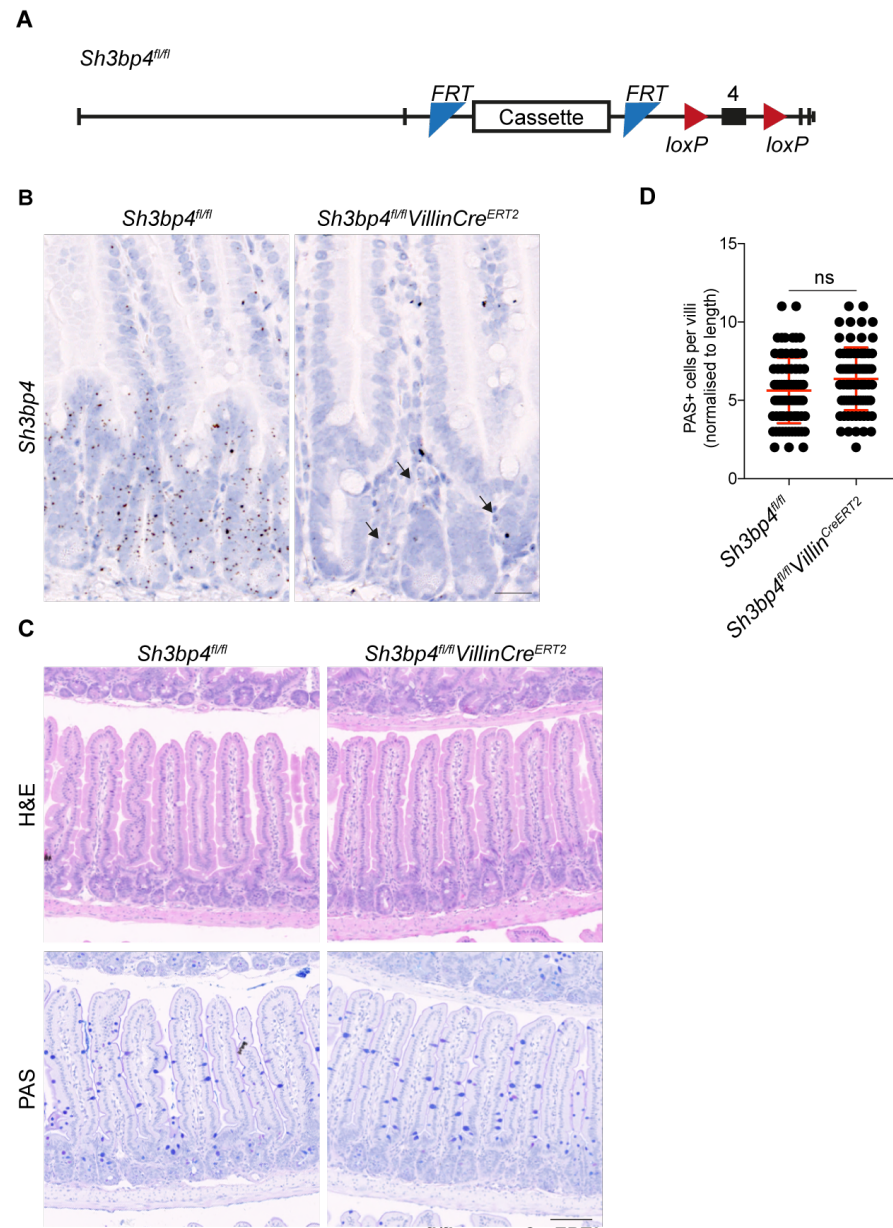


Figure 5.8 Characterisation of *Sh3bp4^{fl/fl} Villin^{CreERT2}* mice 25 days after SH3BP4 loss.

(A) Schematic diagram showing the mouse conditional knock-out strategy. (B) Representative images of *Sh3bp4* RNAscope ISH. RNAscope probe was designed within the flanked region targeting between nucleotides 754 and 1632. *Sh3bp4* expression is lost 25 days after intraperitoneal tamoxifen injection in *Sh3bp4^{fl/fl} Villin^{CreERT2}* (right), whilst expression remains present in the *Sh3bp4^{fl/fl}* control (left). Scale bar, 50µM (C) Representative image of H&E (upper panels) and PAS staining (lower panel) of *Sh3bp4^{fl/fl}* and *Sh3bp4^{fl/fl} Villin^{CreERT2}* intestine (n=4). (D) Quantification of number of PAS⁺ cells per villi in *Sh3bp4^{fl/fl}* and *Sh3bp4^{fl/fl} Villin^{CreERT2}*. PAS⁺ cells were counted in 30 villi per animal. Dots represent the counting for the

Chapter 5. SH3BP4 inhibits intestinal tumour initiation and growth by negative regulation of Wnt signalling

total 120 villi and means \pm SD of the total 4 mice are shown in red. ns - non-significant ($p>0.05$).

Initially, *Sh3bp4* deletion 5 and 15 days after tamoxifen induction did not result in any morphological differences in the intestine (data not shown). We then analysed the intestinal tissues 25 days after *Sh3bp4* deletion and again no obvious defects were detected in tissue morphology or goblet cell differentiation between *Sh3bp4^{fl/fl}Villin^{CreERT2}* and *Sh3bp4^{fl/fl}* (hereafter named as control) animals (Figure 5.8C-D). Despite the lack of morphological differences, intestine from *Sh3bp4^{fl/fl}Villin^{CreER}* mice (25 days after tamoxifen administration) displayed moderately increased expression of the stem cell marker and Wnt target gene *Lgr5* when compared with control mice (Figure 5.9A). The increase in stem cell number was confirmed by increased expression of another stem cell-expressing gene, *Olfm4* (Figure 5.9B-C). On average, *Sh3bp4^{fl/fl}Villin^{CreERT2}* mice contained 8 ± 1 ISCs per crypt compared with 6 ± 1 in control mice. We next asked whether the increase in *Lgr5*-expressing cells was accompanied by an increase in the Paneth cell number. Consistently, a moderate increase in Paneth cell number was observed in *Sh3bp4^{fl/fl}Villin^{CreER}* animals, as revealed by lysozyme staining (**Error! Reference source not found.A**). *Sh3bp4^{fl/fl}Villin^{CreERT2}* mice contained 7 ± 0.2 Paneth cells per crypt compared with 5 ± 0.2 in control mice. Finally, increased proliferation was also observed in the intestinal crypts upon *Sh3bp4* loss (**Error! Reference source not found.B**). The proliferative region on *Sh3bp4^{fl/fl}Villin^{CreERT2}* is on average 121 ± 2.5 μm compared with 94 ± 2.3 μm in control mice. To confirm the increase in proliferation observed in Ki67 staining we will administered EdU 25 days after *Sh3bp4* loss to determine the number of EdU⁺ cells per crypt.

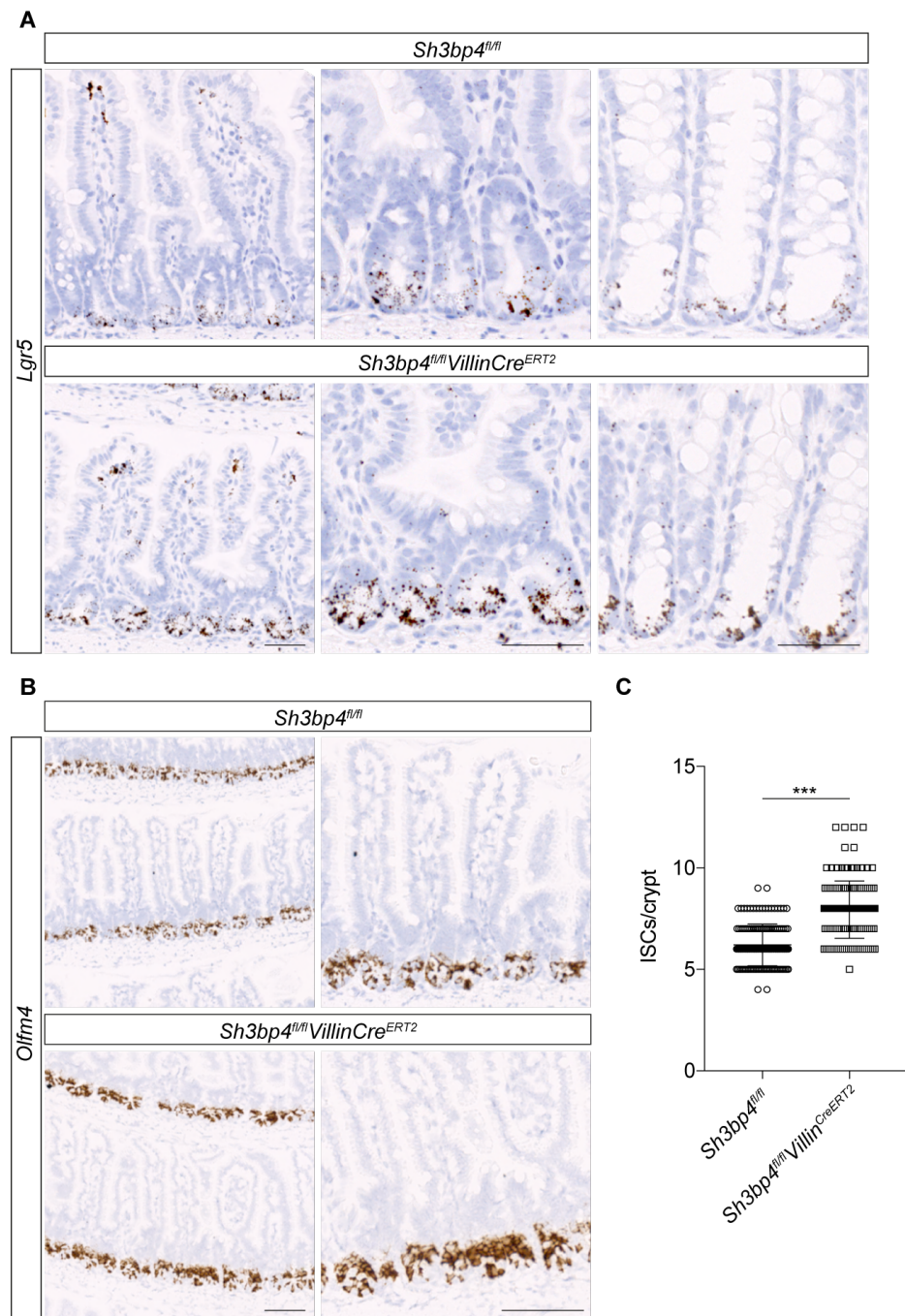


Figure 5.9 Loss of *Sh3bp4* (25 days) increases expression of ISC markers.

Representative image of RNAscope ISH of the Wnt target and stem cell marker, *Lgr5*. Middle panels are magnifications of the crypt region from the small intestine pictures shown on the left. Scale bar, 100µm. Right panel shows colonic crypts, scale bar, 50µm. (B) Representative image of RNAscope ISH of another ISC marker, *Olfr4*. Scale bar, 100µm. Images are representative of 4 animals per condition. (C) Number of *Olfr4* expressing cells per crypt. Dots represent the number of cells in 150 crypts from 3 animals/group (50 crypts/animal). Error bars represent ±SD. ***P<0.001.

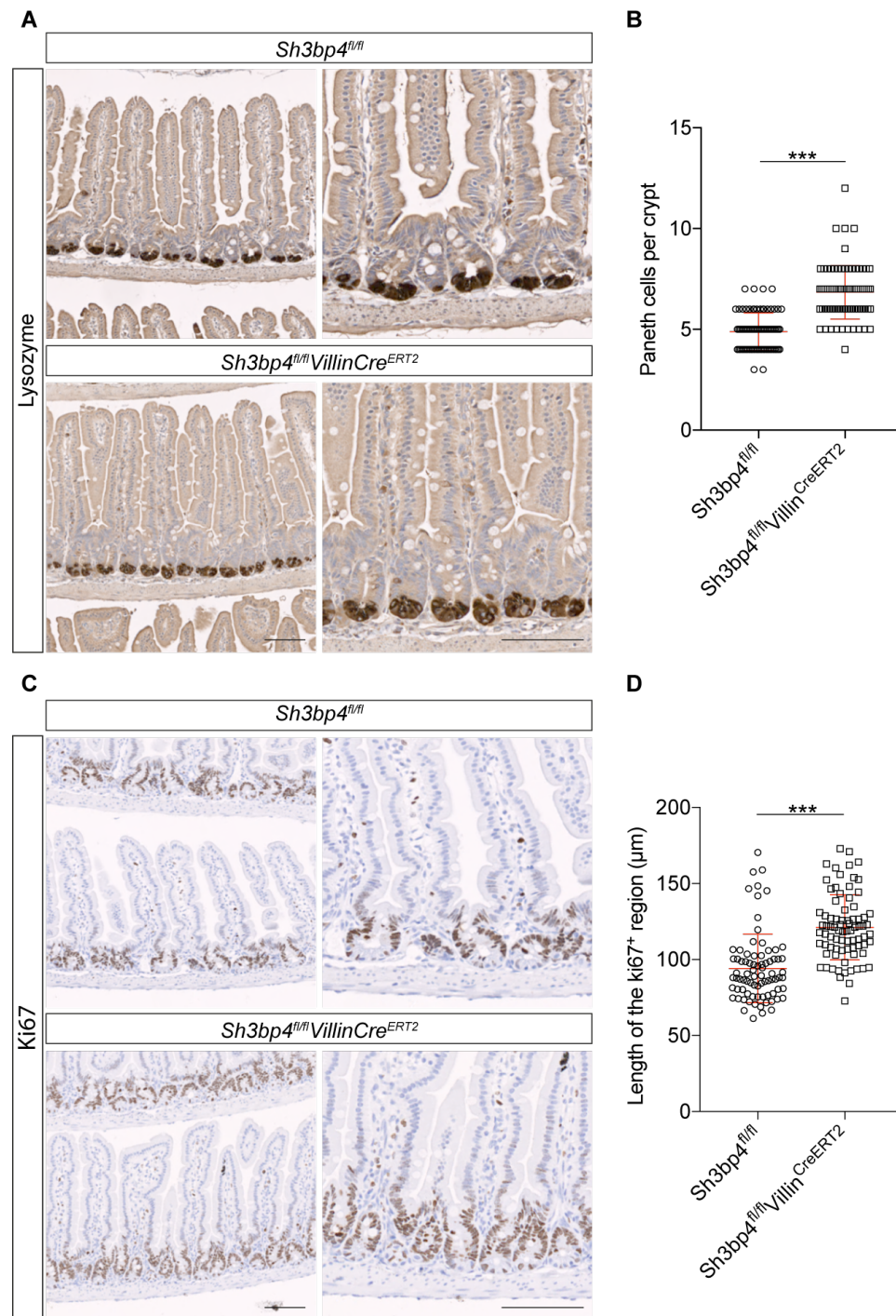


Figure 5.10 *Sh3bp4* deletion (25 days) increases lysozyme expression and crypt proliferation.

(A) Staining for lysozyme to detect Paneth cells. (B) Number of Paneth cells per crypt in *Sh3bp4^{fl/fl}* and *Sh3bp4^{fl/fl} Villin^{CreERT2}*. (C) Ki67⁺ staining in *Sh3bp4^{fl/fl}* and *Sh3bp4^{fl/fl} Villin^{CreERT2}* intestines. (D) Length of the proliferative region measured from the bases of the crypt up to the last detected Ki67⁺ cell. Images on the right are magnifications of crypt regions from small intestine on the left. Images are

representative of 4 animals per condition. Lysozyme positive cells were counted in 20 villi per animal. Dots represent the counting for the total 120 villi and means \pm SD of the total 4 mice are shown in red. *** $P < 0.001$. Scale bar, 100 μ m.

To further analyse if depletion of *Sh3bp4* for a longer period would yield a stronger phenotype, intestinal tissues were analysed 3 months after tamoxifen administration. Interestingly, prolonged *Sh3bp4* loss resulted in longer small intestine and colon than in control animals (Figure 5.11A-B). Ki67 staining also revealed increased proliferation in the *Sh3bp4^{fl/fl}Villin^{CreER}* mice intestines compared with controls (Figure 5.11C). To better assess the increase in proliferation we are now performing EdU staining in *Sh3bp4^{fl/fl}Villin^{CreER}* and *Sh3bp4^{fl/fl}* animals to quantify the number of EdU⁺ cells per crypt. Moreover, we are currently analysing changes in the expression of stem cell markers, Wnt target genes, differentiation and proliferation. Taken together, the results suggest long-term loss of *Sh3bp4* promotes ISC proliferation.

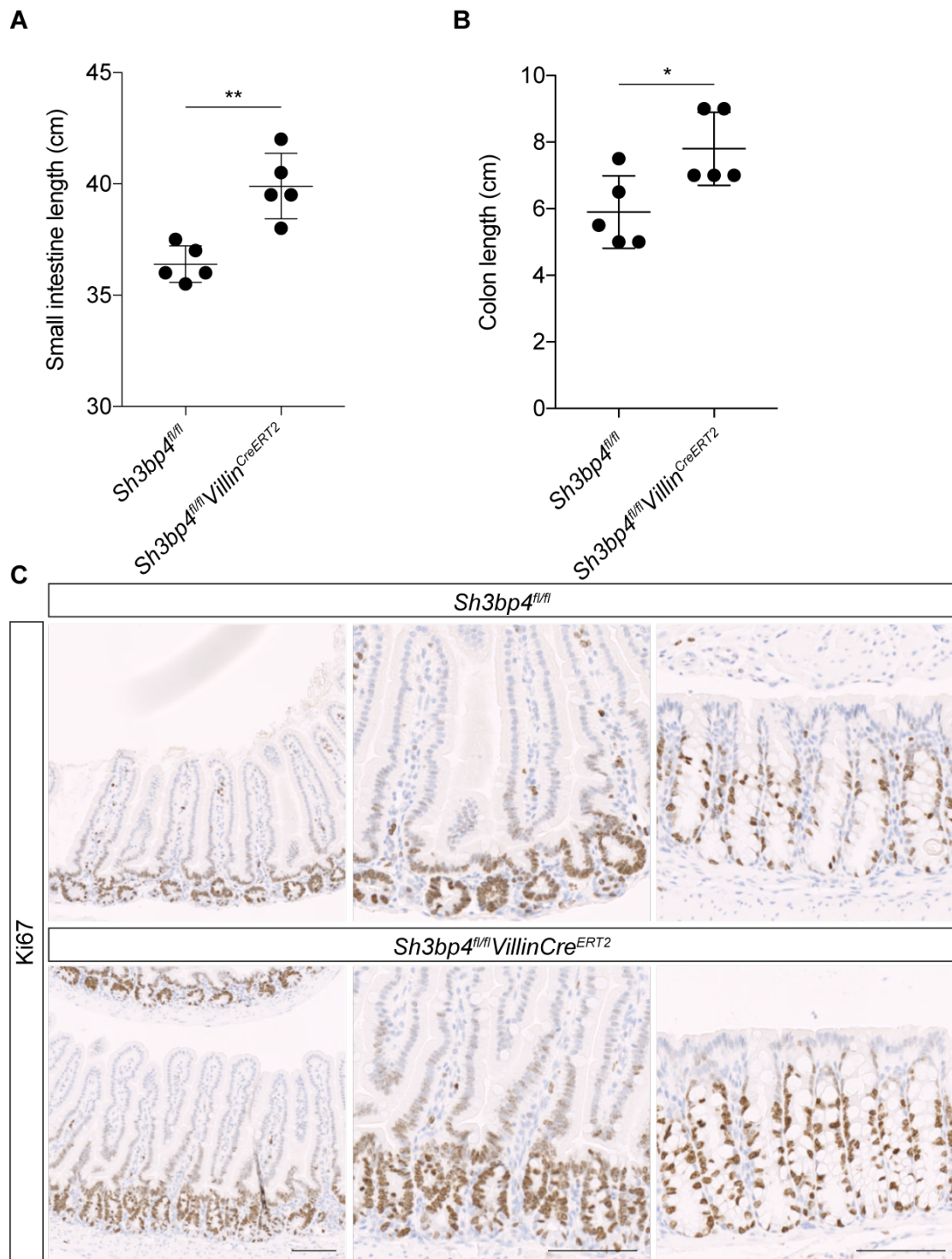


Figure 5.11 Characterisation of *Sh3bp4^{fl/fl} Villin^{CreERT2}* mice 3 months after SH3BP4 loss.

Mean lengths of small intestine (A) and colon (B) from 5 animals per group are shown \pm SD. *P<0.05, **P< 0.01 (C) Ki67 staining of *Sh3bp4^{fl/fl}* and *Sh3bp4^{fl/fl} Villin^{CreERT2}* intestine. Middle panel are magnifications of the crypt region from the small intestine pictures shown on the left. The right panel shows colonic crypts. Scale bar, 100µm.

5.2.5 Loss of SH3BP4 exacerbates *Apc*-mediated intestinal tumour initiation and progression

To study the role of SH3BP4 in intestinal tumorigenesis, *Sh3bp4^{fl/fl}Villin^{CreERT2}* mice were crossed with *Apc^{min}* mice (a mouse model of colon cancer (Li-KuoSu et al., 1992)), to generate *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* mice. Tamoxifen was administered to 6-7-week-old mice and intestinal tissues were analysed 2 months later. Remarkably, *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* mice exhibited almost a 3-fold increase in total adenoma numbers in small intestine compared with control *Apc^{min}* littermates (Figure 5.12A and C). Moreover, despite no significant changes in the mean tumour size, larger tumours were detected in *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* than in *Apc^{min}* animals (Figure 5.12B and C). Tumour development in colon is a rare event in *Apc^{min}* mouse models. Interestingly, colonic tumours were detected in 2 out of the 6 *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* animals analysed, whilst no tumours were found in control *Apc^{min}* mice (Figure 5.12D). In addition, the number of tumours with high grade dysplasia (HGD) in *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* mice is significantly higher when compared to the number of HGD tumours in control *Apc^{min}* animals (Figure 5.12E-F).

Next, we examined the apoptosis in the adenomas. No difference in apoptosis was detected between *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* and *Apc^{min}* mice (Figure 5.13A and B). We will assess the proliferation of these tumours in *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* adenomas by EdU incorporation. PAS staining revealed reduced goblet cell differentiation in the *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* adenomas (Figure 5.14A and C). An average of 78 ± 5 PAS⁺ cells was observed in each *Apc^{min}* adenoma compared with 46 ± 3 in adenomas from *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* animals. On the other hand, an increased number of Paneth cells were observed in *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* adenomas when compared with control *Apc^{min}* animals (Figure 5.14B and D). *Sh3bp4^{fl/fl}Villin^{CreERT2}Apc^{min}* adenomas have 80 ± 4 lysozyme⁺ cells, whilst *Apc^{min}* adenomas show a reduction of 63 ± 3 cells. Further analysis of the intestinal tissues for stem cell markers and Wnt target genes will help to understand if the enhanced tumour phenotype upon SH3BP4 loss is caused by upregulated Wnt signalling and expansion of the ISC population.

Chapter 5. SH3BP4 inhibits intestinal tumour initiation and growth by negative regulation of Wnt signalling

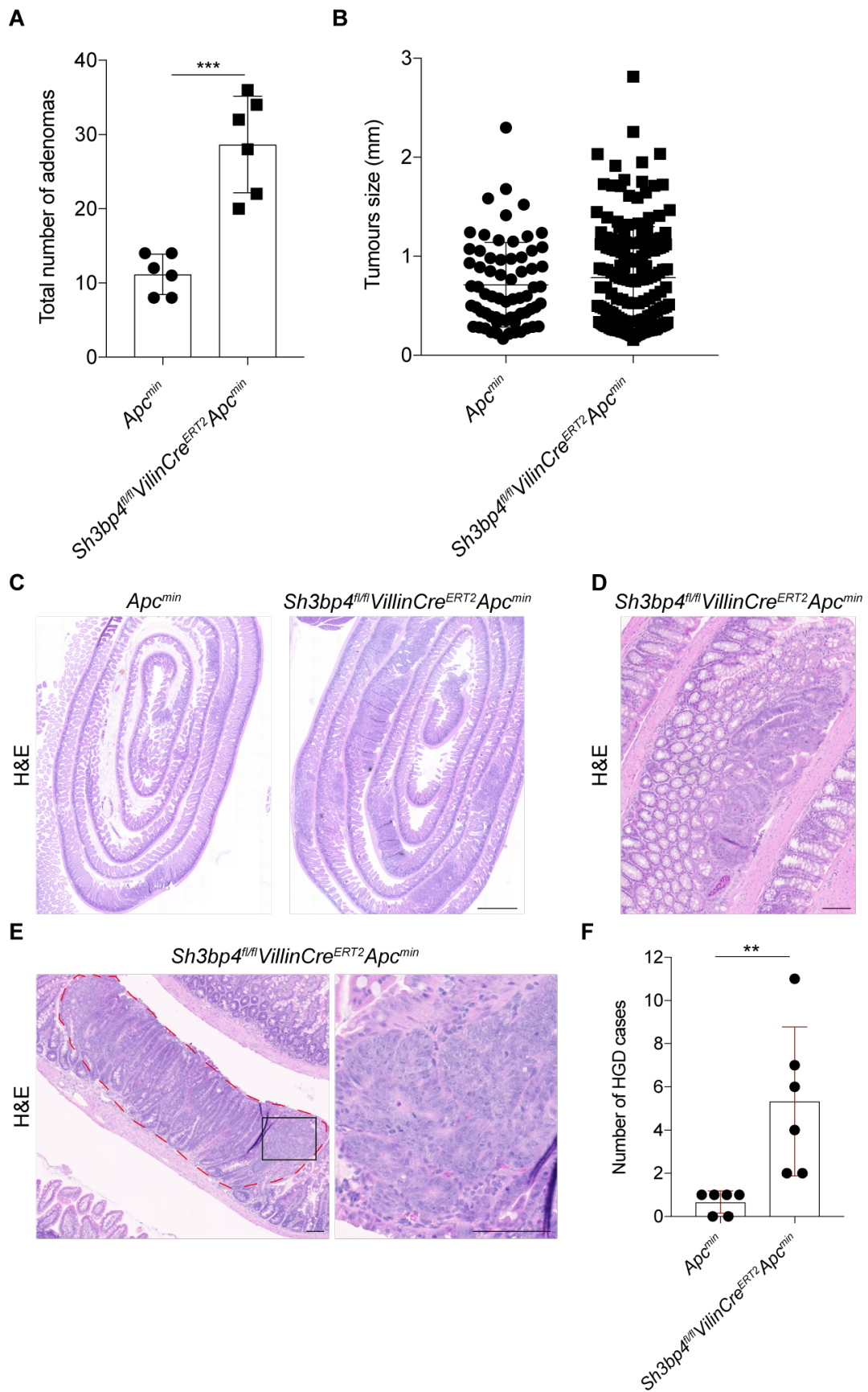


Figure 5.12 Loss of SH3BP4 exacerbates the *Apc^{min}* phenotype.

(A) Total number of adenomas detected in small intestine from 6 animals per group, 2 months after induced-SH3BP4 loss (B) Tumour size distribution observed in *Sh3bp4^{fl/fl} Villin^{CreERT2} Apc^{min}* and *Apc^{min}* mice (C) Representative H&E images of the *Sh3bp4^{fl/fl} Villin^{CreERT2} Apc^{min}* and *Apc^{min}* small intestine. Scale bar, 1mm. (D) H&E stained section of colonic adenoma observed in 2 out of 6 *Sh3bp4^{fl/fl} Villin^{CreERT2} Apc^{min}* animals. (E) Representative H&E staining of high grade dysplasia (HGD) adenoma detected in *Sh3bp4^{fl/fl} Villin^{CreERT2} Apc^{min}* mice. Right panel is a magnification of the highlighted region in the left image. (F) Number of analysed HGD adenomas per animal HGD was defined to be when increased nuclear enlargement and crowding was observed together with disrupted cellular architecture. Scale bar, 100µm. Error bars represent ±SD. ***P < 0.001.

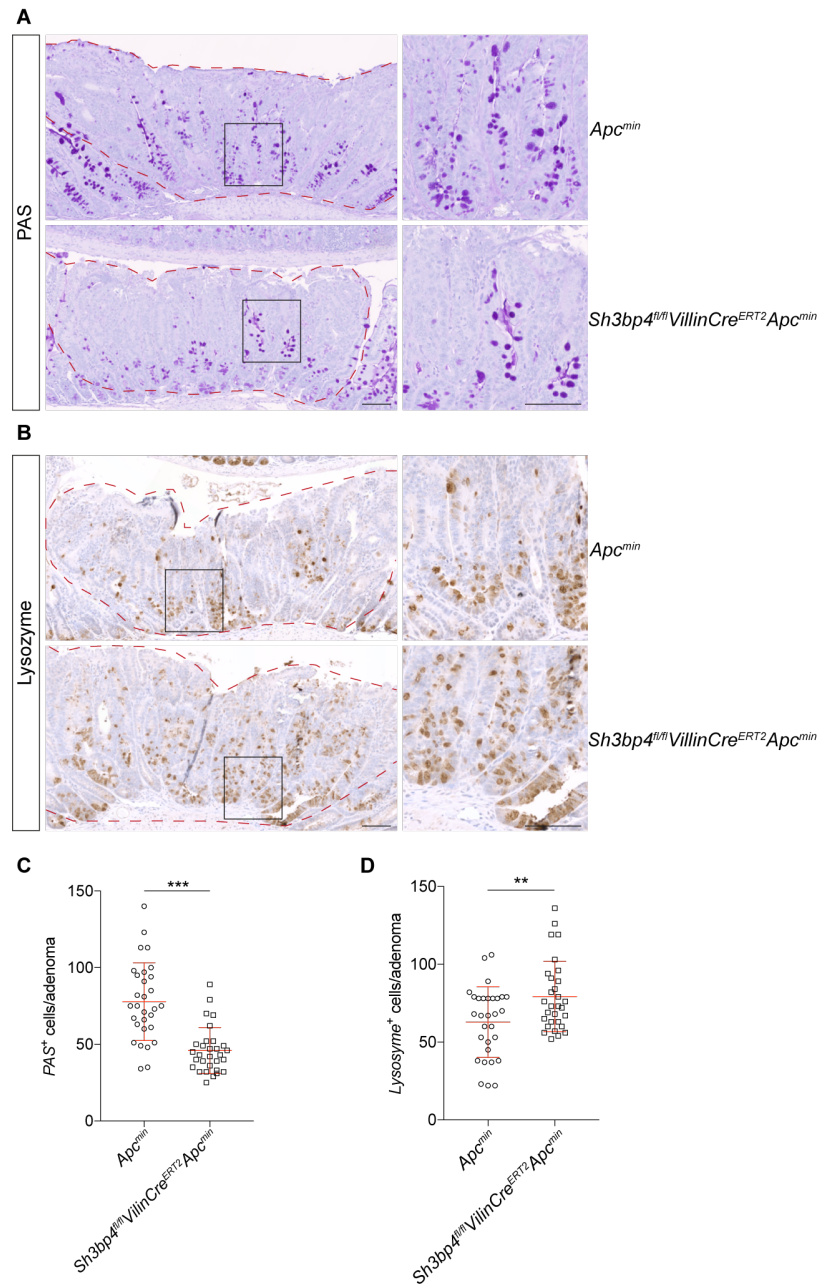


Figure 5.14 Characterisation of goblet cell and Paneth cell differentiation in *Sh3bp4^{fl/fl} Villin^{CreERT2} APC^{min}* mice.

(A) PAS (goblet cells) and lysozyme (Paneth cells) (B) staining was performed on the adenomas of the indicated strains. Right panels are magnifications of the boxed regions of the left-hand side images. Images are representative of 6 animals per condition. Scale bar, 100µm. (C) Number of PAS positive cells and Lysozyme positive cells (D) in adenomas sections from *Sh3bp4^{fl/fl} Villin^{CreERT2} APC^{min}* and *APC^{min}* mice. PAS⁺ and Lysozyme⁺ cells were counted in 5 adenomas with sizes between 0.8 to 1 mm and similar grade of dysplasia, from 6 different animals/group. Dots represent the percentage of positive cells counted in each of the 30 adenomas. Mean ± SD are shown in red. ***P < 0.01, ***P < 0.001.

5.3 Discussion

To the best of our knowledge, this is the first study characterising the role of SH3BP4 in the context of intestine and Wnt/ β -catenin signalling. Our current data show that SH3BP4 inhibits Wnt signalling downstream of the β -catenin destruction complex and is dependent on the ZU5 domain. Loss of SH3BP4 promotes ISC proliferation and tumourigenesis. Taken together the results provide strong evidence in support of our initial hypothesis that SH3BP4 is a negative regulator of Wnt/ β -catenin signalling in intestine.

SH3BP4 functions as a binding protein that is involved in regulation of multiple signalling pathways. In particular, SH3BP4 has been reported to be involved in receptor trafficking (Francavilla et al., 2013; Tosoni et al., 2005), potentially suggesting a regulatory role at the Wnt receptor level. However, the SH3BP4 deletion construct lacking the putative clathrin-binding LIDL motif (SH3BP4 Δ^N) and the SH3_1 mutant (SH3BP4^{W92A}) did not affect its ability to suppress Wnt. Moreover, SH3BP4 was able to inhibit various Wnt activating models downstream of the Wnt receptor level, suggesting that the negative regulatory role of SH3BP4 in Wnt/ β -catenin signalling is unlikely to be via recycling or internalisation of the Wnt receptors. In addition, SH3BP4 has also been shown to be a negative regulator of the mTOR pathway (Kim et al., 2012b). To test whether Wnt signal activation induced by SH3BP4 depletion is caused by activation of mTOR signalling, we treated the Δ SH3BP4 cells with the mTOR inhibitor rapamycin. Inhibition of mTOR signalling did not suppress Wnt activation in the Δ SH3BP4 cells, suggesting that the negative regulatory role of SH3BP4 in Wnt/ β -catenin signalling is independent of the mTOR pathway. Importantly, the SH3_1 domains that mediate the interaction with Rag GTPases for mTOR inhibition is not required for Wnt signalling suppression (Kim et al., 2012b), whilst ZU5 is the critical domain for Wnt signal inhibition. The data support the notion that SH3BP4 negatively regulates both Wnt and mTOR pathways independently.

Our preliminary data suggests that SH3BP4 regulates Wnt/ β -catenin signalling at the nucleus, possibly via interactions with β -catenin and/or APC proteins. Interestingly, APC has been reported to antagonise β -catenin transcriptional activity

by promoting its proteosomal degradation (Orford et al., 1997), nuclear export (Henderson, 2000) and by interfering with the β -catenin/TCF complex formation (Hamada and Bienz, 2004). Since SH3BP4 is predominantly localised at the nucleus and perinuclear membrane, we hypothesised that SH3BP4 may function to facilitate APC-mediated nuclear export of β -catenin or interfere with β -catenin/TCF transcriptional complex formation. Further experiments are required to further investigate the Wnt negative regulatory role of SH3BP4 at the nucleus and how precisely the ZU5 domain is involved.

Thus far, our data show that the Wnt negative regulator SH3BP4 is expressed at the intestinal crypt bottom and is co-localised with the ISC marker *Lgr5*. We therefore proceeded to characterise its functional implication during normal intestinal homeostasis. Despite normal histology, 25 days after SH3BP4 loss, a moderate increase in *Lgr5*- and *Olfm4*-expressing cells was observed in intestinal tissues, as well as enhanced proliferation. In addition, animals analysed 3 months after SH3BP4 loss had significantly longer small intestines and colons. Taken together, the data suggest that *Sh3bp4* plays a crucial role in ISC maintenance. We are currently analysing the crypt-villus structures of these animals as well as the expression of Wnt targets, stem cell and differentiation markers. It will be important to determine whether expansion of the pool of ISCs upon *Sh3bp4* deletion is caused by upregulated Wnt signalling, and if cell differentiation is affected or not. Of note, intestinal homeostasis was only affected after prolonged SH3BP4 loss alone, suggesting that loss of the Wnt negative regulator SH3BP4 may result in increased tumour susceptibility, possibly due to the increase in basal Wnt signalling within the crypt compartment. Further investigation of intestinal tissues over longer periods of SH3BP4 loss will be important to study its tumorigenic potential. We have already induced deletion of *Sh3bp4* in a cohort of animals and will collect the intestine for analysis after approximately 1 year.

In addition to the regulatory role in intestinal homeostasis, our data further show that loss of SH3BP4 augments the *Apc*^{min} intestinal tumour phenotype by promoting both tumour initiation and progression. Together, our study uncovers the tumour suppressive role of SH3BP4 in the intestine for the first time by modulating Wnt signalling/ β -catenin.

Chapter 6. General discussion

Over the past few decades, remarkable insights have been obtained into the molecular events underlying the development of CRC. Large scale whole-genome sequencing studies have resulted in detailed molecular landscapes of driver events and revealed a complex picture of key players in CRC. This large amount of data has certainly advanced our collective understanding of CRC and facilitated therapeutic development. The Wnt signalling pathway plays a crucial role in both intestinal homeostasis and CRC development. In addition to the well characterised central players in the Wnt signalling cascade, more recently other novel regulators involved in fine-tuning the pathway for ISC maintenance have been identified. Many of these have also been reported to be deregulated in CRC, reinforcing the crucial link between intestinal homeostasis and tumourigenesis in the context of Wnt signalling. The aim of my PhD project was to identify and characterise novel Wnt regulators for a better understanding of the mechanisms that regulate ISC homeostasis and the corresponding tumour biology. By analysing the publicly available genome-wide transcriptomic data from TCGA (TCGA, 2012), we identified a “Stem-Cell/Wnt cluster” that is enriched with many stem cell markers, Wnt target genes and/or Wnt inhibitors. We hypothesised that some of the genes within this cluster are Wnt inhibitors responsible for a negative feedback loop. In particular, we have uncovered two novel Wnt negative regulators: ZAK and SH3BP4. Our data show that both proteins are responsible for generating a Wnt negative feedback loop to refine Wnt/ β -catenin signalling levels during tissue homeostasis, regeneration and tumourigenesis.

6.1 Wnt/ β -catenin negative feedback mechanisms in the ISC compartment

Wnt signal activation at the bottom of intestinal crypts is crucial for ISC maintenance. Yet, hyperactivation of the pathway can lead to pathological dysplasia due to uncontrolled ISC expansion (Figure 6.1). Thus, precise control of the Wnt

signalling level within the ISC compartment is vital for intestinal homeostasis. To fine-tune Wnt activity within the intestinal crypts, several negative Wnt regulators are transcriptionally regulated by β -catenin/TCF directly to create a negative feedback loop to antagonise WNT signalling. These inhibitors act at different subcellular levels of the signalling cascade to regulate important intracellular or extracellular components. For instance, AXIN2 and RNF43 are both reported to be Wnt targets expressed by stem cells that antagonise Wnt signalling at the destruction complex and Frizzled receptor levels, respectively. Here we show, for the first time, that both ZAK and SH3BP4 are negative Wnt regulators expressed by stem cells. Deletion of *Zak* enhances radiation-induced intestinal regeneration, whilst SH3BP4 loss promotes ISC proliferation and augments the *Apc*^{min} tumour phenotype (Figure 6.1). Our findings highlight the crucial role of the negative feedback mechanisms required to regulate key cellular processes during tissue homeostasis, such as stem cell self-renewal, proliferation and differentiation.

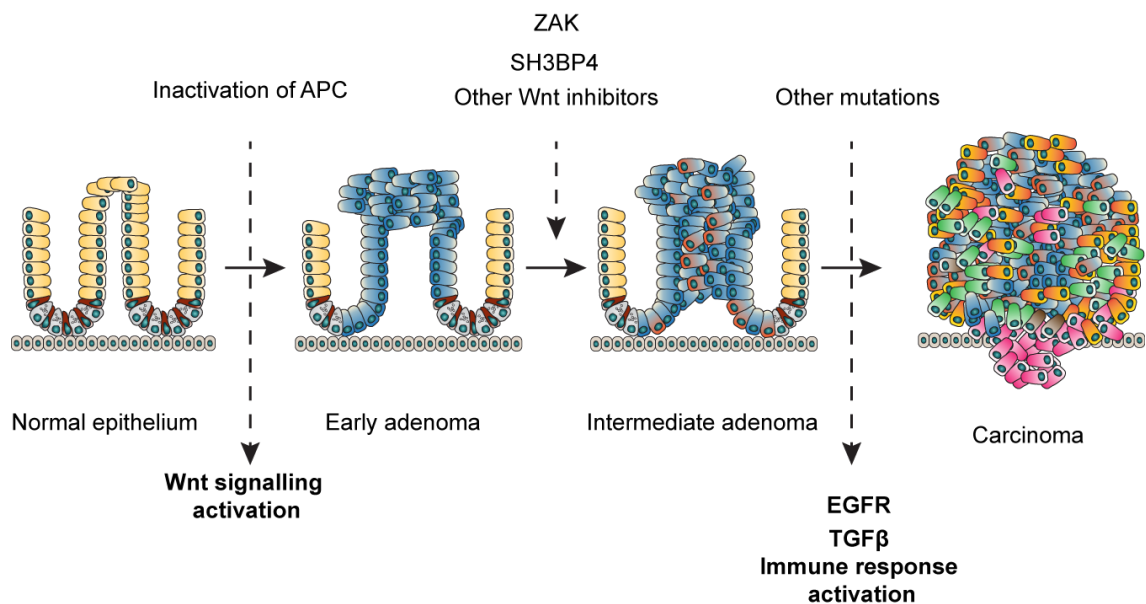


Figure 6.1 Wnt inhibitors and CRC.

Loss of APC function occurs in 80% of CRC patients and results in activation of Wnt signalling and development of early adenomas. Accumulation of mutations in components of the Wnt signalling negative feedback mechanism could lead to increase in the grade of dysplasia as we have shown to be the case for SH3BP4 in the *Apc*-mutated background. Accumulation of mutations in other proteins like TP53, SMAD4, KRAS, and PI3K result in cancer progression to carcinoma.

It has been previously reported that crypt stem cells are the origin of intestinal cancer (Barker et al., 2009). Therefore, it is possible that mutations of Wnt inhibitors expressed by stem cells could potentiate the transition from stem cell to cancer stem cell. In fact, recurrent mutations of *AXIN2* and *RNF43* have been previously reported in CRCs (Giannakis et al., 2014; TCGA, 2012). As mentioned in chapter 3, recent sequencing studies with a larger cohort of CRCs showed the presence of mutation in *SH3BP4* and *ZAK* in CRCs. The mutations are distributed across the whole gene without any hotspot. The majority of the mutations are missense mutations, although two truncations are reported in both genes. One might expect a higher frequency of mutation in Wnt inhibitors than the one observed for *ZAK* and *SH3BP4*. There are several possibilities reasons that could explained a low number of mutations in Wnt inhibitors in CRC. A potential reason is the principle of random replacement of ISCs, known as neutral drift. A ISC in a crypt can be replaced by any other ISC (Lopez-Garcia et al., 2010; Snippert et al., 2010b), which could result in mutation loss. Secondly, despite the fact that mutations in a particular gene could give selective advantage to the carrying cell, genes can play a role in multiple cellular pathways and be selected accordingly to the overall effect in the different pathways. Finally, it is possible that, to find “the just-right” Wnt signalling level, as it has been suggested for APC function (Fodde et al., 2001), cancer cells may favour the maintenance of some negative feedback responses that confer the “just-right” expression of a subset of genes that provide a growth advantage to the cancer cell.

Many studies of transcriptomic or tissue microarray analysis in various CRC cohorts have been described, and have revealed differential expression of genes or proteins between different samples. Upregulation of certain genes or proteins in a subpopulation of CRCs relative to normal tissues are often interpreted as putative oncogenes. Contrary to this dogma, our findings suggest that this is not necessarily the case. The “Stem-Cell/Wnt cluster” identified from our analysis indeed shows an overall upregulated gene expression in MSS-CRCs. However, rather than being oncogenes, many of the genes in this cluster are in fact Wnt inhibitors expressed by stem cells. The upregulated expression is simply a consequence of being Wnt/ β -catenin transcriptional targets leading to abnormally highly expression in *APC*-mutated CRCs. On the contrary, many of the genes are indeed downregulated in non-*APC* mutated CRCs. In fact, many Wnt negative regulators such as *AXIN2*,

NOTUM, RNF43 and MULE have all been previously reported to be upregulated in intestinal tumours (Dominguez-Brauer et al., 2016; Koo et al., 2012; Lustig et al., 2002; Robertis et al., 2015), although they are considered tumour suppressors rather than oncogenes. Similarly, ZAK has also previously been reported to be upregulated in CRCs (Liu et al., 2014b). Here we propose that both ZAK and SH3BP4 are Wnt negative regulators expressed by stem cells and function as growth/tumour suppressors.

6.2 Mechanistic role of ZAK and SH3BP4 and their therapeutic implications

Activation of Wnt signalling in the majority of CRCs is via *APC* mutations. Therefore, therapeutic interventions targeting Wnt components upstream of the β -catenin destruction complex would not be efficient for this subtype of CRCs. The most effective targets would be downstream of the cytoplasmic β -catenin destruction complex. However, despite extensive efforts, these targets have proven to be difficult to identify (Antas et al., 2015). Here, we identified two novel Wnt negative regulators, ZAK and SH3BP4, that function to control Wnt/ β -catenin signalling at the “right levels”. Interestingly, both genes are able to suppress Wnt signalling in *APC*-mutated cells. Characterising the Wnt inhibitory mechanism of the two genes may shed light on approaches to develop therapeutic interventions.

ZAK. Our data show that ZAK inhibits Wnt/ β -catenin signalling downstream of β -catenin phosphorylation and ubiquitination and is kinase-dependent. Cytoplasmic expression of ZAK suggests that its Wnt inhibitory role is unlikely to act at the β -catenin/TCF transcriptional level. Previous studies have shown that inhibiting tankyrase-dependent degradation of AXIN is able to inhibit Wnt/ β -catenin signalling in *APC*-mutated CRC cells and suppress tumour growth (Waler et al., 2012; Waler et al., 2011). The data suggest that stabilising AXIN can indeed inhibit Wnt signalling downstream of the defective β -catenin destruction complex. AXIN is considered to be the rate-limiting factor for β -catenin degradation (Salic et al., 2000), whilst

phosphorylation of AXIN is important for the regulation of the destruction complex (MacDonald et al., 2009). Together, we hypothesise that ZAK may function to stabilise AXIN, thereby promoting β -catenin degradation (Figure 6.2). On the other hand, ZAK may also function to retain β -catenin in the cytoplasm via an unknown mechanism, thereby inhibiting β -catenin/TCF complex formation. Further investigation will be needed to elucidate the inhibitory role of ZAK in Wnt pathway.

Our *in vivo* data show that *Zak* deletion enhances intestinal regeneration, whilst its tumourigenic potential has not yet been addressed. Interestingly, ZAK has been reported as off-target of several clinically approved kinase inhibitors including anticancer drugs (Karaman et al., 2008; Vin et al., 2014a; Vin et al., 2014b; Wong et al., 2013). For example, off-target inhibition of ZAK by BRAF inhibitors has been associated with secondary cutaneous squamous cell carcinoma (cSSC) in patients treated for melanoma (Vin et al., 2014b). In particular, vemurafenib/PLX4720 suppresses apoptosis through off-target inhibition of ZAK. Our data further demonstrate that ZAK inhibition could activate Wnt/ β -catenin signalling, highlighting the risks associated of using these anticancer drugs with off-target activity against ZAK.

As mentioned in chapter 4, *Zak*^{-/-} animals generated for this project were shared with two different laboratories which have reported to us that ZAK seems to be important to muscle physiology and to normal function of the endoplasmic reticulum (ER). Disturbance in the ER environment causes several stress responses such as oxidative stress and DNA damage, long known to be involved in cancer biology (Urrea et al., 2016). This phenotype is now being explored by our collaborators to better understand the physiological significance of ZAK protein in cellular stress response.

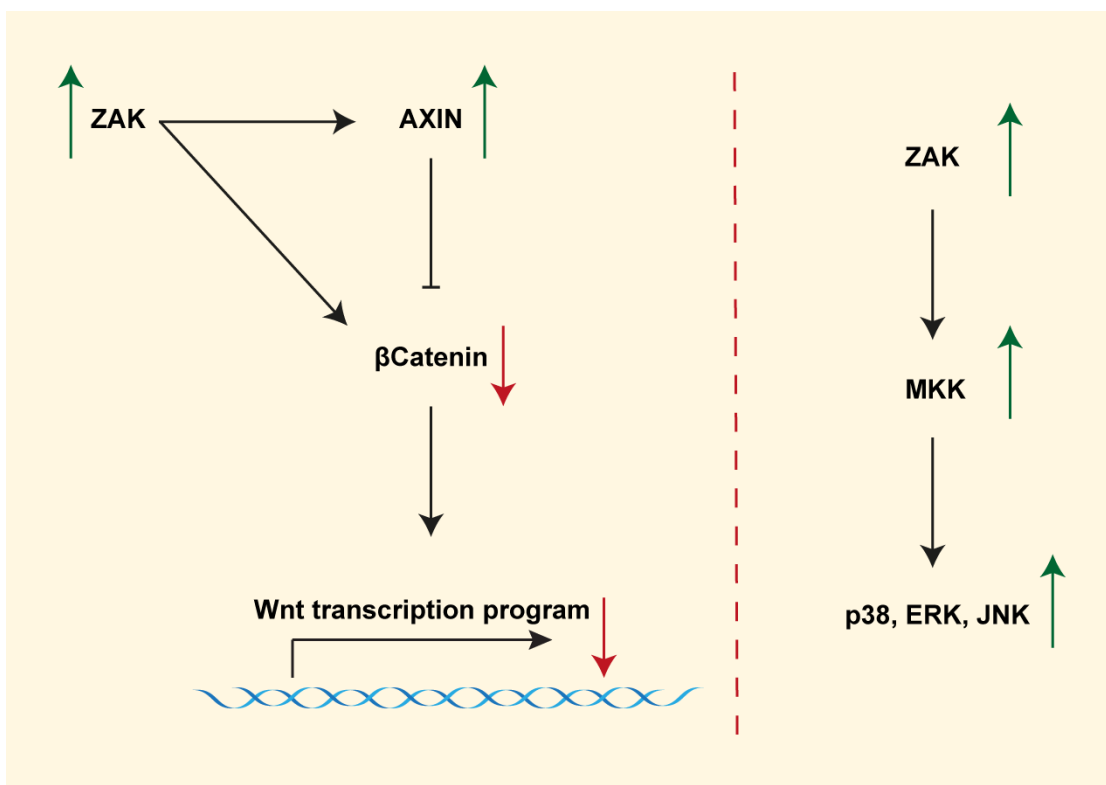


Figure 6.2 ZAK inhibits Wnt signalling independently of MAPK pathway activation.

ZAK inhibits Wnt/ β -catenin signalling downstream of β -catenin phosphorylation and ubiquitination. We hypothesise that ZAK may function to stabilise AXIN, thereby promoting β -catenin degradation. On the other hand, ZAK may also function to retain β -catenin in the cytoplasm via an unknown mechanism, thereby inhibiting β -catenin/TCF complex formation. Green arrowa indicate increase in protein levels or protein activity. ZAK can activate ERK, p38 and JNK pathways. Our data suggests that the Wnt inhibitory role of ZAK is MAPK-independent. Red arrows indicate decrease in protein levels or activity.

SH3BP4 also inhibits Wnt/ β -catenin signalling downstream of β -catenin phosphorylation and ubiquitination. Unlike ZAK, SH3BP4 is strongly localised in the nucleus and physically interacts with APC and β -catenin. It is plausible that SH3BP4 plays a role in APC-mediated nuclear shuttling of β -catenin (Figure 6.3). Indeed, a study from Henderson has shown that the N-terminal half of APC contains two active nuclear export sequences (NES) (Henderson, 2000), which is consistent with the fact that SH3BP4 can inhibit Wnt/ β -catenin signalling in our HEK293T Δ APC cells with intact NES. Further studies will help characterise the potential role of SH3BP4 in APC-mediated β -catenin nuclear shuttling. Understanding the full mechanism by

which β -catenin is exported from the nucleus or how the β -catenin/TCF complex could be regulated could shed light into new therapeutic strategies against CRC. Moreover, we have attempted to identify the specific domain of SH3BP4 that is responsible for Wnt signal inhibition. To do so, different SH3BP4 deletion/mutant constructs lacking various SH3BP4 domains were generated. The construct lacking the ZU5 domain failed to exert full Wnt inhibitory function. This data suggests that the inhibitory role of SH3BP4 is dependent on the ZU5 domain, a domain poorly characterized. It is plausible that this domain is involved in the interaction of SH3BP4 with APC or β -catenin (Figure 6.3). Further experiments are required to assess that.

SH3BP4 has previously been shown to play a regulatory role in clathrin-mediated internalisation of membrane receptors such as transferrin receptor and fibroblast growth factor receptor (Francavilla et al., 2013; Tosoni et al., 2005). Our data shows that expression of SH3BP4 is able to inhibit TCF-TopFLASH reporter activity induced by the constitutively active form of LRP6 receptor (ΔN -LRP6), suggesting that the inhibitory role of SH3BP4 in Wnt signalling is downstream of Wnt ligand receptors. Moreover, the SH3BP4 deletion construct lacking the putative clathrin-binding LIDL motif (SH3BP4 ΔN) did not affect its ability to suppress Wnt signalling.

SH3BP4 is also a negative regulator of mTORC1 signalling. SH3BP4 binds to inactive GDP-bound Rag GTPase via the SH3 domain and inhibits the formation of the active Rag GTPase complex, inactivating mTOR signalling (Kim et al., 2012b). On the other hand, previous studies have shown that Apc mutated intestinal tumours are dependent on mTORC1 signalling for tumour growth (Faller et al., 2014; Fujishita et al., 2008). These facts raise the question whether increase in the number of ISCs after *Sh3bp4* loss is a consequence of the synergistic effect of both Wnt and mTOR signalling activation. Our *in vitro* data suggest that SH3BP4-mediated Wnt inhibition is mTOR-independent. To isolate the contribution of which pathway to the observed phenotype, we will be treating *Sh3bp4^{fl/fl} Villin^{CreERT2}* and *Sh3bp4^{fl/fl}* animals with the mTOR inhibitor rapamycin, and assess changes in proliferation and Wnt target genes expression after *Sh3bp4* loss.

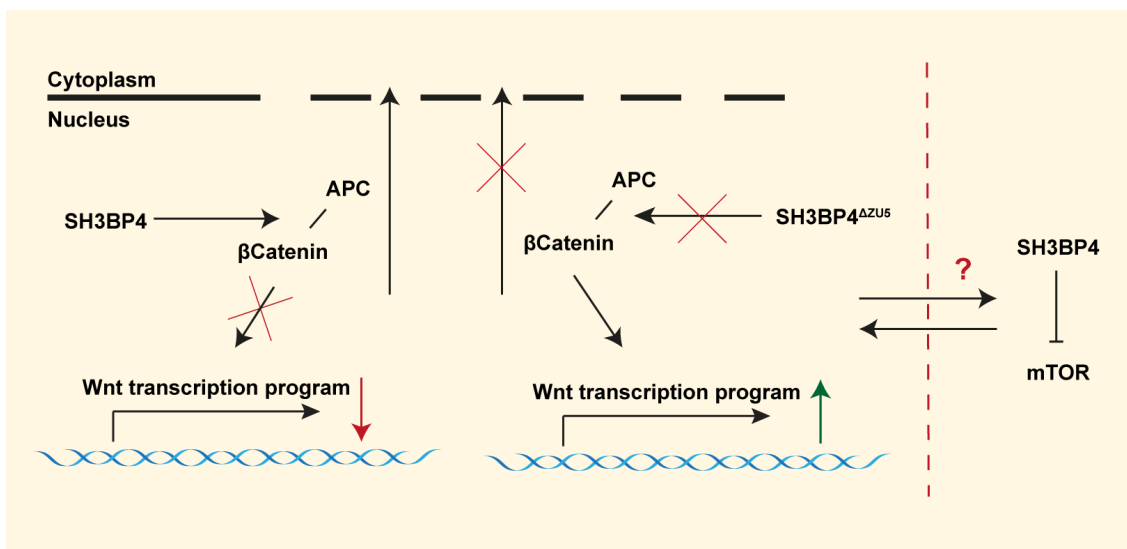


Figure 6.3 SH3BP4 inhibitory role in Wnt signalling.

SH3BP4 inhibits Wnt/ β -catenin signalling downstream of β -catenin phosphorylation and ubiquitination. SH3BP4 is strongly localised in the nucleus and physically interacts with APC and β -catenin. We hypothesise that SH3BP4 plays a role in APC-mediated nuclear shuttling of β -catenin, thereby reducing the levels of nuclear β -catenin that can bind to the TCF complex and active Wnt transcription program. ZU5 domain is fundamental for the inhibitory role of SH3BP4 in Wnt signalling. We believe that this domain is involved in the interaction of SH3BP4 with APC or β -catenin. SH3BP4 also negative regulates mTORC1 signalling. It is plausible that the phenotype observed in mice intestine after *Sh3bp4* loss is a consequence of the synergistic effect of both Wnt and mTOR signalling activation.

6.3 Future directions

In addition to the molecular mechanism, further investigation will be needed for functional characterisation *in vivo*. In fact, organoids are a suitable model for *ex vivo* functional studies of Wnt signalling activation, tumour transformation and regeneration. Normal intestinal organoids are well differentiated with budding structures recapitulating crypt-villus architecture *in vivo*, whilst *Apc*-mutated organoids show remain cystic and display a non-budding phenotype as a result of Wnt-activated ISC hyperproliferation. It would be interesting to examine whether expression of ZAK and SH3BP4 in *Apc*-mutated organoids would restore the budding structure in spheroids as a result of Wnt signalling inhibition.

As previously mentioned, ZAK has two isoforms distinguished by their unique C-terminal protein sequence and these have been shown to be differentially expressed in normal and tumour tissues (Liu et al., 2014b). This raises the question

of whether the two isoforms perform unique or overlapping roles during intestinal homeostasis.

Our preliminary data suggest that the Wnt inhibitory role of ZAK is MAPK-independent. Although the MAPK inhibitors have been widely used in other studies, the efficiency of the inhibitors should be confirmed in our system by examining changes in phosphorylation of MAPKs. Moreover, treatments of combined MAPK inhibitors should be considered due to the compensatory role of different MAPKs.

As mentioned in chapter 4, we do not know if deletion of *Zak* accelerates regeneration of the intestinal epithelium or if *Zak-null* animals are more resistant to damage-induced radiation. We will have to analyse the intestine of induced-injury mice at earlier time points to answer this question. In addition, we need to further investigate the expression of Wnt target genes and stem-cell markers in normal *Zak*^{-/-} mice. In addition, it would be important to further characterise the role of ZAK loss in intestinal tumorigenesis in addition to tissue regeneration. We are currently breeding the *Zak*^{-/-}*Apc*^{min} mouse strain for further analyses.

SH3BP4 is expressed in the intestinal crypts overlapping with the ISC marker *Lgr5*. To test if stem-cell specific loss of SH3BP4 alters intestinal homeostasis, we have generated another mouse strain that allows deletion of *Sh3bp4* specifically in ISCs (*Sh3bp4*^{fl/fl}*Lgr5*^{CreERT2}). The mice are currently being analysed for markers of stem cell, proliferation and differentiation.

We have not studied the isolated contribution of SH3BP4 deficiency to tumorigenesis. To analyse if loss of SH3BP4 alone is sufficient to promote tumorigenesis, we will analyse a cohort of animals one year after *Sh3bp4* loss.

In this project, *Apc*^{min} mice were used as a model for studying intestinal tumourigenesis. However, given the hypothesis that deregulation of ZAK and SH3BP4 is an alternative Wnt activating mechanism in CRC, it would be interesting to study the tumourigenic roles of ZAK and SH3BP4 using another intestinal tumour model. For instance, the inflammation-related cancer model induced by dextran sodium sulphate (DSS) and azoxymethane (AOM) will induce colon-specific lesions, which may represent a better CRC model for ZAK and SH3BP4.

The progression of CRC from an early adenoma to carcinoma is accompanied by a set of mutations including *KRAS*, *SMAD4*, *TP53*. Identifying genes whose loss exerts a selective pressure favouring other mutations rate to facilitate adenoma

tumorigenesis are of extreme importance. It would be worth investigating whether loss of ZAK or SH3BP4 favours CRC-associated mutations.

With the advent of new technologies to examine thousands of genes in parallel, the true complexity of CRC has become evident. This big amount of data allows us to reassess the nature of CRCs knowledge and it is clear that we still lack a deeper understanding of CRC pathology. If cancer is a disease in which genes that control cell division are mutated, thus causing cellular overgrowth, why targeted inhibitors of cell division fail to cure CRCs? If aberrant Wnt signalling activation is the main cause of CRCs initiation, why multiple inhibitors that suppress Wnt fail to cure most of CRCs patients? (see our review Antas et al., 2015). In addition, we now know that heterogeneity across individual and tumour microenvironment add further challenges to the understanding of CRC biology. Every study represents an opportunity to refine our knowledge of this disease. Here, we highlight the crucial role of the Wnt negative feedback mechanism in intestinal homeostasis. We have identified two novel Wnt target genes expressed by stem cells, *Sh3bp4* and *Zak*, which act as Wnt inhibitors to fine-tune the levels of Wnt/ β -catenin signalling during normal tissue maintenance (Figure 6.4). This is the first time that ZAK and SH3BP4 have been implicated in intestinal homeostasis and cancer via Wnt/ β -catenin signalling.

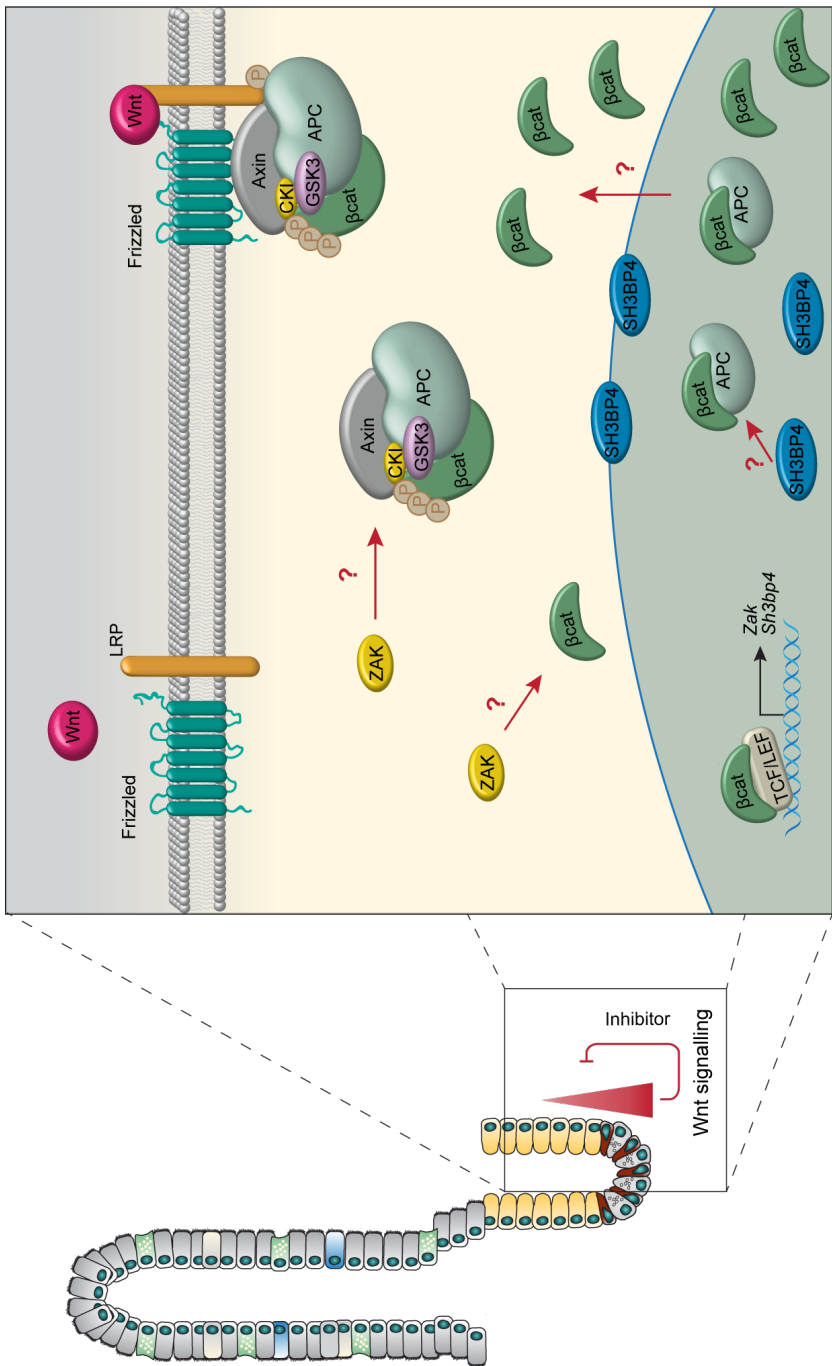


Figure 6.4 Graphical summary of potential mechanisms of action.

Wnt signalling is the main driving force of ISC self-renewal and intestinal homeostasis at the bottom of the intestinal crypts. To maintain the “just-right” level of Wnt signalling, inhibitors such as ZAK and SH3BP4 are transcriptionally regulated by β-catenin/TCF to function as a negative feedback mechanism. ZAK is expressed mainly in the cytoplasm and could potentially regulate Wnt/β-catenin signalling either by stabilising AXIN or by interfering with the levels of nuclear β-catenin. On the other hand, SH3BP4 is expressed predominantly in the nucleus and may modulate β-catenin nuclear export by regulating the β-catenin/APC complex.

Chapter 7. Appendix 1

Mutations in CRISPR/CAS9 targeted HEK293T cells

HEK293T Δ ZAK clone

- Mutation 2 - 189bp insertion sequence

TCGAGCCAAATGGATATGCGTGTTTCAGTCACGTGATCAGGGCCTACTGAGCT
GCGATTTTCCTGTCTGCTCAGCTGCTTGACAATTGCCAGTTGAGCTGTGGACC
ATGATTACGCCAAGCTCTAGCTAGAGGTCGACGGTATACATTTAGGTGACACT
ATAGAATACTCAAGCTTATGCATGCGGCCGCATCTAGAGTCGACCAGTCACAG
 G

- Mutation 1- predicted protein sequence of truncated form (88aa)

MSSLGASFVQISSTTCSFLKTAVEEVLGVCIEPNGQGGGCKEVTQNRERGRNPE
 RPQS QKHHPVLWSDSGTSQLWHRHRICFPGLPV**Stop**

- Mutation 2 - predicted protein sequence of truncated form (42aa)

MSSLGASFVQISSTTCSFLKTAVEEVLGVCIEPNGYACSVT**Stop**

HEK293T Δ SH3BP4 clone

- Mutation 1- predicted protein sequence of truncated form (110aa)

MAAQRIRAANSNGLPRCKSEGTLIDLSEGFSETSFNDIKVSPSPALLVDNPTPFGN
 AKEVIAIKDYCPTNFTTLKFSKGDHSWTHLAVSGGTHHTPPKWATSPPPMCSP**Stop**
 p

- Mutation 2 - predicted protein sequence of truncated form (89aa)

MAAQRIRAANSNGLPRCKSEGTLIDLSEGFSETSFNDIKVSPSPALLVDNPTPFGN
 AK EVIAIKDYCPTNFTTLKFSKGDHLYGHIWR**Stop**

Reference List

- Abaco, G.M.D., Whitehead, R.H., and Burgess, A.W. (1996). Synergy between Apc min and an activated ras mutation is sufficient to induce colon carcinomas. *Molecular Cell Biology* 16, 884-891.
- Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* 16, 3797-3804.
- Akiyama, H., Lyons, J.P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., Deng, J.M., Taketo, M.M., Nakamura, T., Behringer, R.R., *et al.* (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes & Development* 18, 1072-1087.
- Amit, S., Hatzubai, A., Birman, Y., Andersen, J.S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y., and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of 'beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes & Development* 16, 1066-1076.
- Antas, P., Novellademunt, L., and Li, V.S. (2015). Targeting Wnt signaling in colorectal cancer. A Review in the Theme: Cell Signaling: Proteins, Pathways and Mechanisms. *Am J Physiol Cell Physiol* 309, C511-521.
- Anvarian, Z., Nojima, H., van Kappel, E.C., Madl, T., Spit, M., Viertler, M., Jordens, I., Low, T.Y., van Scherpenzeel, R.C., Kuper, I., *et al.* (2016). Axin cancer mutants form nanoaggregates to rewire the Wnt signaling network. *Nat Struct Mol Biol* 23, 324-332.
- Assie, G., Letouze, E., Fassnacht, M., Jouinot, A., Luscap, W., Barreau, O., Omeiri, H., Rodriguez, S., Perlemoine, K., Rene-Corail, F., *et al.* (2014). Integrated genomic characterization of adrenocortical carcinoma. *Nature genetics* 46, 607-612.
- Avsian-Kretchmer, O., and Hsueh, A.J.W. (2004). Comparative Genomic Analysis of the Eight-Membered Ring Cystine Knot-Containing Bone Morphogenetic Protein Antagonists. *Molecular Endocrinology* 18, 1-12.
- Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., and Clevers, H. (2001). The chromatin remodelling factor Brg-1 interacts with β -catenin to promote target gene activation. *The EMBO journal* 20, 4935-4943.
- Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., Sansom, O.J., and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 457, 608-611.
- Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., *et al.* (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449, 1003-1007.
- Basak, O., Beumer, J., Wiebrands, K., Seno, H., van Oudenaarden, A., and Clevers, H. (2016). Induced Quiescence of Lgr5+ Stem Cells in Intestinal Organoids Enables Differentiation of Hormone-Producing Enteroendocrine Cells. *Cell stem cell* 20, 177–190.
- Bass, A.J., Lawrence, M.S., Brace, L.E., Ramos, A.H., Drier, Y., Cibulskis, K., Sougnez, C., Voet, D., Saksena, G., Sivachenko, A., *et al.* (2011). Genomic sequencing of

colorectal adenocarcinomas identifies a recurrent VTI1A-TCF7L2 fusion. *Nature genetics* 43, 964-968.

Bastide, P., Darido, C., Pannequin, J., Kist, R., Robine, S., Marty-Double, C., Bibeau, F., Scherer, G., Joubert, D., Hollande, F., *et al.* (2007). Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. *J Cell Biol* 178, 635-648.

Battle, E., Henderson, J.T., Beghtel, H., Born, M.M.W.v.d., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., Wetering, M.v.d., Pawson, T., *et al.* (2002). β -Catenin and TCF Mediate Cell Positioning in the Intestinal Epithelium by Controlling the Expression of EphB/EphrinB. *Cell* 111, 251-263.

Behrens, J., Jerchow, B.-A., Würtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kühl, M., Wedlich, D., and Birchmeier, W. (1998). Functional Interaction of an Axin Homolog, Conductin, with β -Catenin, APC, and GSK3 β . *Science* 280, 596-599.

Beumer, J., and Clevers, H. (2016). Regulation and plasticity of intestinal stem cells during homeostasis and regeneration. *Development* 143, 3639-3649.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.-C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature* 382, 225 - 230

Bilić, J., Huang, Y.-L., Davidson, G., Zimmermann, T., Cruciat, C.-M., Bienz, M., and Niehrs, C. (2007). Wnt Induces LRP6 Signalosomes and Promotes Dishevelled-Dependent LRP6 Phosphorylation. *Science* 316, 1619-1622.

Bjerknes M, C.H. (1999). Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* 116, 7-14.

Blache, P., van de Wetering, M., Duluc, I., Domon, C., Berta, P., Freund, J.N., Clevers, H., and Jay, P. (2004). SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J Cell Biol* 166, 37-47.

Blahnik, K.R., Dou, L., O'Geen, H., McPhillips, T., Xu, X., Cao, A.R., Iyengar, S., Nicolet, C.M., Ludascher, B., Korf, I., *et al.* (2010). Sole-Search: an integrated analysis program for peak detection and functional annotation using ChIP-seq data. *Nucleic Acids Res* 38, 1-17.

Bottomly, D., Kyler, S.L., McWeeney, S.K., and Yochum, G.S. (2010). Identification of beta-catenin binding regions in colon cancer cells using ChIP-Seq. *Nucleic Acids Res* 38, 5735-5745.

Buczacki, S.J.A., Zecchini, H.I., Nicholson, A.M., Russell, R., Vermeulen, L., Kemp, R., and Winton, D.J. (2013). Intestinal label-retaining cells are secretory precursors expressing Lgr5. *Nature* 495, 65-69.

Buechling, T., Chaudhary, V., Spirohn, K., Weiss, M., and Boutros, M. (2011). p24 proteins are required for secretion of Wnt ligands. *EMBO Rep* 12, 1265-1272.

Cadigan, K.M., and Waterman, M.L. (2012). TCF/LEFs and Wnt signaling in the nucleus. *Cold Spring Harbor perspectives in biology* 4, 1-22.

- Cai, B., Chang, S.H., Becker, E.B., Bonni, A., and Xia, Z. (2006). p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65. *The Journal of biological chemistry* 281, 25215-25222.
- Carmon, K.S., Gong, X., Lin, Q., Thomas, A., and Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/ β -catenin signaling. *Proceedings of the National Academy of Sciences of the United States of America* 108, 11452-11457.
- Castanon, I., Abrami, L., Holtzer, L., Heisenberg, C.P., van der Goot, F.G., and Gonzalez-Gaitan, M. (2013). Anthrax toxin receptor 2a controls mitotic spindle positioning. *Nat Cell Biol* 15, 28-39.
- Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* 395, 604-608.
- Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., *et al.* (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2, 401-404.
- Cervantes, S., Yamaguchi, T.P., and Hebrok, M. (2009). Wnt5a is essential for intestinal elongation in mice. *Developmental biology* 326, 285-294.
- Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature* 410, 37-40.
- Chen, C.M., Orefice, L.L., Chiu, S.L., LeGates, T.A., Hattar, S., Huganir, R.L., Zhao, H., Xu, B., and Kuruvilla, R. (2017). Wnt5a is essential for hippocampal dendritic maintenance and spatial learning and memory in adult mice. *Proceedings of the National Academy of Sciences of the United States of America* 114, 619-628.
- Cheng, H., and Leblond, C.P. (1974a). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine I. Columnar cell. *American Journal of Anatomy* 141, 461-479.
- Cheng, H., and Leblond, C.P. (1974b). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *American Journal of Anatomy* 141, 461-479.
- Cho, Y.Y., Bode, A.M., Mizuno, H., Choi, B.Y., Choi, H.S., and Dong, Z. (2004). A novel role for mixed-lineage kinase-like mitogen-activated protein triple kinase α in neoplastic transformation and tumor development. *Cancer research* 64, 3855-3864.
- Choi, H.S., Choi, B.Y., Cho, Y.Y., Zhu, F., Bode, A.M., and Dong, Z. (2005). Phosphorylation of Ser28 in histone H3 mediated by mixed lineage kinase-like mitogen-activated protein triple kinase alpha. *The Journal of biological chemistry* 280, 13545-13553.
- Civenni, G., Holbro, T., and Hynes, N.E. (2003). Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells. *EMBO Reports* 4, 166-171.

- Clevers, H. (2006). Wnt/ β -catenin signaling in development and disease. *Cell* 127, 469-480.
- Clevers, H., and Nusse, R. (2012). Wnt/ β -catenin signaling and disease. *Cell* 149, 1192-1205.
- Collins, R.T., and Treisman, J.E. (2000). Osa-containing Brahma chromatin remodeling complexes are required for the repression of Wingless target genes. *Genes & Development* 14, 3140-3152.
- Cong, F., and Varmus, H. (2004). Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of β -catenin. *Proceedings of the National Academy of Sciences of the United States of America* 110, 2882-2887.
- Coombs, G.S., Yu, J., Canning, C.A., Veltri, C.A., Covey, T.M., Cheong, J.K., Utomo, V., Banerjee, N., Zhang, Z.H., Jadulco, R.C., *et al.* (2010). WLS-dependent secretion of WNT3A requires Ser209 acylation and vacuolar acidification. *J Cell Sci* 123, 3357-3367.
- Coudreuse, D.Y.M., Roël, G., Betist, M.C., Destrée, O., and Korswagen, H.C. (2006). Wnt Gradient Formation Requires Retromer Function in Wnt-Producing Cells. *Science* 312 921-924.
- Cruciat, C.M., and Niehrs, C. (2013). Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harbor perspectives in biology* 5, 1-26.
- Cuilliere-Dartigues, P., El-Bchiri, J., Krimi, A., Buhard, O., Fontanges, P., Flejou, J.F., Hamelin, R., and Duval, A. (2006). TCF-4 isoforms absent in TCF-4 mutated MSI-H colorectal cancer cells colocalize with nuclear CtBP and repress TCF-4-mediated transcription. *Oncogene* 25, 4441-4448.
- Dalerba, P., Dylla, S.J., Park, I.-K., Liu, R., Wang, X., Cho, R.W., Hoey, T., Gurney, A., Huang, E.H., Simeone, D.M., *et al.* (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 104, 10158-10163.
- Dann, C.E., Hsieh, J.C., Rattner, A., Sharma, D., Nathans, J., and Leahy, D.J. (2001). Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. *Nature* 412, 86-90.
- Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A., and Niehrs, C. (2005). Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* 438, 867-872.
- Davis, H., Irshad, S., Bansal, M., Rafferty, H., Boitsova, T., Bardella, C., Jaeger, E., Lewis, A., Freeman-Mills, L., Giner, F.C., *et al.* (2015). Aberrant epithelial GREM1 expression initiates colonic tumorigenesis from cells outside the stem cell niche. *Nature Medicine* 21, 62-70.
- de Jong, P.R., Taniguchi, K., Harris, A.R., Bertin, S., Takahashi, N., Duong, J., Campos, A.D., Powis, G., Corr, M., Karin, M., *et al.* (2016). ERK5 signalling rescues intestinal epithelial turnover and tumour cell proliferation upon ERK1/2 abrogation. *Nature communications* 7, 1-15.

- de Lau, W., Barker, N., Low, T.Y., Koo, B.K., Li, V.S., Teunissen, H., Kujala, P., Haegebarth, A., Peters, P.J., van de Wetering, M., *et al.* (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* 476, 293-297.
- de Sousa e Melo, F., Kurtova, A.V., Harnoss, J.M., Kljavin, N., Hoeck, J.D., Hung, J., Anderson, J.E., Storm, E.E., Modrusan, Z., Koeppen, H., *et al.* (2017). A distinct role for Lgr5+ stem cells in primary and metastatic colon cancer. *Nature* 543, 676-680.
- De Sousa, E.M.F., Wang, X., Jansen, M., Fessler, E., Trinh, A., de Rooij, L.P., de Jong, J.H., de Boer, O.J., van Leersum, R., Bijlsma, M.F., *et al.* (2013). Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* 19, 614-618.
- Deng, C., Reddy, P., Cheng, Y., Luo, C.W., Hsiao, C.L., and Hsueh, A.J. (2013). Multi-functional norrin is a ligand for the LGR4 receptor. *J Cell Sci* 126, 2060-2068.
- Depeille, P., Henricks, L.M., Ven, R.A.H.v.d., Lemmens, E., Wang, C.-Y., Matli, M., Werb, Z., Haigis, K.M., Donner, D., Warren, R., *et al.* (2015). RasGRP1 opposes proliferative EGFR–SOS1–Ras signals and restricts intestinal epithelial cell growth. *Nature Cell Biology* 17, 804-815.
- Dissanayake, S.K., Wade, M., Johnson, C.E., O'Connell, M.P., Leotlela, P.D., French, A.D., Shah, K.V., Hewitt, K.J., Rosenthal, D.T., Indig, F.E., *et al.* (2007). The Wnt5A/Protein Kinase C Pathway Mediates Motility in Melanoma Cells via the Inhibition of Metastasis Suppressors and Initiation of an Epithelial to Mesenchymal Transition. *The Journal of biological chemistry* 282, 17259-17271.
- Doble, B.W., Patel, S., Wood, G.A., Kockeritz, L.K., and Woodgett, J.R. (2007). Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev Cell* 12, 957-971.
- Doerks, T., Copley, R.R., Schultz, J., Ponting, C.P., and Bork, P. (2002). Systematic identification of novel protein domain families associated with nuclear functions. *Genome Research* 12, 47-56.
- Dominguez-Brauer, C., Hao, Z., Elia, A.J., Fortin, J.M., Nechanitzky, R., Brauer, P.M., Sheng, Y., Mana, M.D., Chio, I., Haight, J., *et al.* (2016). Mule Regulates the Intestinal Stem Cell Niche via the Wnt Pathway and Targets EphB3 for Proteasomal and Lysosomal Degradation. *Cell stem cell* 19, 205-216.
- Durand, A., Donahue, B., Peignon, G., Letourneur, F., Cagnard, N., Slomianny, C., Perret, C., Shroyer, N.F., and Romagnolo, B. (2012). Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor Math1 (Atoh1). *Proceedings of the National Academy of Sciences of the United States of America* 109, 8965–8970.
- Duval, A., Gayet, J., Zhou, X.P., Iacopetta, B., Thomas, G., and Hamelin, R. (1999). Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability. *Cancer research* 59, 4213-4215.
- el Marjou, F., Janssen, K.P., Chang, B.H., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* 39, 186-193.

- Erpel, T., Superti-Furga, G., and Courtneidge, S.A. (1995). Mutational Analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. *The EMBO Journal* *14*, 963-975.
- Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S.J., *et al.* (2000). Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer research* *60*, 4366-4371.
- Fagotto, F.o., Glück, U., and Gumbiner, B.M. (1998). Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of β -catenin. *Cell* *8*, 181-190.
- Faller, W.J., Jackson, T.J., Knight, J.R., Ridgway, R.A., Jamieson, T., Karim, S.A., Jones, C., Radulescu, S., Huels, D.J., Myant, K.B., *et al.* (2014). mTORC1-mediated translational elongation limits intestinal tumour initiation and growth. *Nature* *517*, 497–500.
- Farin, H.F., Van Es, J.H., and Clevers, H. (2012). Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology* *143*, 1518-1529.
- Fearon, E., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* *61*, 759-767.
- Fearon, E.R. (2011). Molecular genetics of colorectal cancer. *Annual review of pathology* *6*, 479-507.
- Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J.W., Comber, H., Forman, D., and Bray, F. (2013). Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* *49*, 1374-1403.
- Fiedler, M., Graeb, M., Mieszczanek, J., Rutherford, T.J., Johnson, C.M., and Bienz, M. (2015). An ancient Pygo-dependent Wnt enhanceosome integrated by Chip/LDB-SSDP. *eLife* *4*, 1-22.
- Fiedler, M., Mendoza-Topaz, C., Rutherford, T.J., Mieszczanek, J., and Bienz, M. (2011). Dishevelled interacts with the DIX domain polymerization interface of Axin to interfere with its function in down-regulating β -catenin. *Proceedings of the National Academy of Sciences of the United States of America* *108*, 1937–1942.
- Firestein, R., Bass, A.J., Kim, S.Y., Dunn, I.F., Silver, S.J., Guney, I., Freed, E., Ligon, A.H., Vena, N., Ogino, S., *et al.* (2008). CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. *Nature* *455*, 547-551.
- Flack, J.E., Mieszczanek, J., Novcic, N., and Bienz, M. (2017). Wnt-Dependent Inactivation of the Groucho/TLE Co- repressor by the HECT E3 Ubiquitin Ligase Hyd/UBR5. *Molecular cell* *67*, 181–193.
- Fodde, R., Smits, R., and Clevers, H. (2001). APC, signal transduction and genetic instability in colorectal cancer. *Nature Reviews Cancer* *1*.

- Francavilla, C., Rigbolt, K.T., Emdal, K.B., Carraro, G., Vernet, E., Bekker-Jensen, D.B., Streicher, W., Wikstrom, M., Sundstrom, M., Bellusci, S., *et al.* (2013). Functional proteomics defines the molecular switch underlying FGF receptor trafficking and cellular outputs. *Molecular cell* 51, 707-722.
- Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., and Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. *Nature* 435, 964-968.
- Fre, S., Pallavi, S.K., Huyghe, M., Lae, M., Janssen, K.P., Robine, S., Artavanis-Tsakonas, S., and Louvard, D. (2009). Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. *Proceedings of the National Academy of Sciences of the United States of America* 106, 6309-6314.
- Freeman, M. (2000). Feedback control of intracellular signalling in development. *Nature* 408, 313-319.
- Freeman, M., and Bienz, M. (2001). EGF receptor/Rolled MAP kinase signalling protects cells against activated Armadillo in the Drosophila eye. *EMBO reports* 2, 157-162.
- Fu, X., Li, L., and Peng, Y. (2012). Wnt signalling pathway in the serrated neoplastic pathway of the colorectum: possible roles and epigenetic regulatory mechanisms. *Journal of clinical pathology* 65, 675-679.
- Fujishita, T., Aoki, K., Lane, H.A., Aoki, M., and Taketo, M.M. (2008). Inhibition of the mTORC1 pathway suppresses intestinal polyp formation and reduces mortality in ApcDelta716 mice. *Proceedings of the National Academy of Sciences of the United States of America* 105, 13544-13549.
- Fukuda, T., Chen, L., Endo, T., Tang, L., Lu, D., Castro, J.E., Widhopf, G.F., Rassenti, L.Z., Cantwell, M.J., Prussak, C.E., *et al.* (2008). Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. *Proceedings of the National Academy of Sciences of the United States of America* 105, 3047-3052.
- Furukawa, T., Kuboki, Y., Tanji, E., Yoshida, S., Hatori, T., Yamamoto, M., Shibata, N., Shimizu, K., Kamatani, N., and Shiratori, K. (2011). Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. *Scientific Reports* 1, 1-7.
- Galiatsatos, P., and Foulkes, W.D. (2006). Familial adenomatous polyposis. *Am J Gastroenterol* 101, 385-398.
- Gallo, K.A., and Johnson, G.L. (2002). Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nature reviews Molecular cell biology* 3, 663-672.
- Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M.A., Andre, P., Robinson, J., Sood, R., Minami, Y., *et al.* (2011). Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. *Developmental Cell* 20, 163-176.

- Gao, C., Cao, W., Bao, L., Zuo, W., Xie, G., Cai, T., Fu, W., Zhang, J., Wu, W., Zhang, X., *et al.* (2010). Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. *Nature Cell Biology* 12, 781-790.
- Gerbe, F., Legraverend, C., and Jay, P. (2012). The intestinal epithelium tuft cells: specification and function. *Cell and Molecular Life Sciences* 69, 2907-2917.
- Giannakis, M., Hodis, E., Jasmine Mu, X., Yamauchi, M., Rosenbluh, J., Cibulskis, K., Saksena, G., Lawrence, M.S., Qian, Z.R., Nishihara, R., *et al.* (2014). RNF43 is frequently mutated in colorectal and endometrial cancers. *Nature genetics* 46, 1264-1266.
- Giráldez, A.J., Copley, R.R., and Cohen, S.M. (2002). HSPG Modification by the Secreted Enzyme Notum Shapes the Wingless Morphogen Gradient. *Cell* 2, 667-676.
- Glinka, A., Dolde, C., Kirsch, N., Huang, Y.L., Kazanskaya, O., Ingelfinger, D., Boutros, M., Cruciat, C.M., and Niehrs, C. (2011). LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. *EMBO Rep* 12, 1055-1061.
- Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* 391, 357-362.
- Gotoh, I., Adachi, M., and Nishida, E. (2001). Identification and characterization of a novel MAP kinase kinase kinase, MLTK. *The Journal of biological chemistry* 276, 4276-4286.
- Green, J., Nusse, R., and van Amerongen, R. (2014). The role of Ryk and Ror receptor tyrosine kinases in Wnt signal transduction. *Cold Spring Harbor perspectives in biology* 6.
- Gregorieff, A., Pinto, D., Begthel, H., Destree, O., Kielman, M., and Clevers, H. (2005). Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology* 129, 626-638.
- Gross, E.A., Callow, M.G., Waldbaum, L., Thomas, S., and Ruggieri, R. (2002). MRK, a mixed lineage kinase-related molecule that plays a role in gamma-radiation-induced cell cycle arrest. *The Journal of biological chemistry* 277, 13873-13882.
- Gross, J.C., Chaudhary, V., Bartscherer, K., and Boutros, M. (2012). Active Wnt proteins are secreted on exosomes. *Nat Cell Biol* 14, 1036-1045.
- Grumolato, L., Liu, G., Mong, P., Mudbhary, R., Biswas, R., Arroyave, R., Vijayakumar, S., Economides, A.N., and Aaronson, S.A. (2010). Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. *Genes Dev* 24, 2517-2530.
- Guinney, J., Dienstmann, R., Wang, X., de Reynies, A., Schlicker, A., Soneson, C., Marisa, L., Roepman, P., Nyamundanda, G., Angelino, P., *et al.* (2015). The consensus molecular subtypes of colorectal cancer. *Nature Medicine* 21, 1350-1356.
- Gupta, J., Del Barco Barrantes, I., Igea, A., Sakellariou, S., Pateras, I.S., Gorgoulis, V.G., and Nebreda, A.R. (2014). Dual Function of p38alpha MAPK in Colon Cancer:

- Suppression of Colitis-Associated Tumor Initiation but Requirement for Cancer Cell Survival. *Cancer cell* 25, 484-500.
- Habas, R., Kato, Y., and He, X. (2001). Wnt/Frizzled Activation of Rho Regulates Vertebrate Gastrulation and Requires a Novel Formin Homology Protein Daam1. *Cell* 107, 843–854.
- Hall, P.A., Coates, P.J., Ansari, B., and Hopwood, D. (1994). Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *Journal of Cell Science* 107, 3569-3577
- Hamada, F., and Bienz, M. (2004). The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. *Dev Cell* 7, 677-685.
- Hao, H.X., Xie, Y., Zhang, Y., Charlat, O., Oster, E., Avello, M., Lei, H., Mickanin, C., Liu, D., Ruffner, H., *et al.* (2012). ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature* 485, 195-200.
- Haramis, A.P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G.J., and Clevers, H. (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* 303, 1684-1686.
- Hatzis, P., van der Flier, L.G., van Driel, M.A., Guryev, V., Nielsen, F., Denissov, S., Nijman, I.J., Koster, J., Santo, E.E., Welboren, W., *et al.* (2008). Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. *Molecular and cellular biology* 28, 2732-2744.
- He, X.C., Zhang, J., Tong, W.G., Tawfik, O., Ross, J., Scoville, D.H., Tian, Q., Zeng, X., He, X., Wiedemann, L.M., *et al.* (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nature genetics* 36, 1117-1121.
- Heath, J.P. (1996). Epithelial cell migration in the intestine. *Cell biology international* 20, 139-146.
- Heisenberg, C.-P., Tada, M., Rauch, G.-J., Saúde, L., Concha, M.L., Geisler, R., Stemple, D.L., Smith, J.C., and Wilson, S.W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation . *Nature* 405, 76-81.
- Henderson, B.R. (2000). Nuclear-cytoplasmic shuttling of APC regulates b-catenin subcellular localization and turnover. *Nature Cell Biology* 2, 653-660.
- Hernández, A.R., Klein, A.M., and Kirschner, M.W. (2012). Kinetic Responses of b-Catenin Specify the Sites of Wnt Control. *Science* 338, 1337-1340.
- Heuberger, J., Kosel, F., Qi, J., Grossmann, K.S., Rajewsky, K., and Birchmeier, W. (2014). Shp2/MAPK signaling controls goblet/paneth cell fate decisions in the intestine. *Proceedings of the National Academy of Sciences of the United States of America* 111, 3472-3477.

- Hoang, B., Moos, M., Vukicevic, J.S., and Luyten, F.P. (1996). Primary Structure and Tissue Distribution of FRZB, a Novel Protein Related to Drosophila Frizzled, Suggest a Role in Skeletal Morphogenesis. *The Journal of biological chemistry* 271, 26131-26131.
- Holstein, T.W. (2012). The evolution of the Wnt pathway. *Cold Spring Harbor perspectives in biology* 4, 1-17.
- Houde, M., Laprise, P., Jean, D., Blais, M., Asselin, C., and Rivard, N. (2001). Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox transcription factor CDX2. *The Journal of biological chemistry* 276, 21885-21894.
- Howe, J.R., Bair, J.L., Sayed, M.G., Anderson, M.E., Mitros, F.A., Petersen, G.M., Velculescu, V.E., Traverso, G., and Vogelstein, B. (2001). Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. *Nature genetics* 28, 184-187.
- Howe, J.R., Roth, S., Ringold, J.C., Summers, R.W., Järvinen, H.J., Sistonen, P., Tomlinson, I.P.M., Houlston, R.S., Bevan, S., Mitros, F.A., *et al.* (1998). Mutations in the SMAD4/DPC4 Gene in Juvenile Polyposis. *Science* 280, 1086-1088.
- Hua Tian, B.B., Warming, S., Leong, K.G., Rangell, L., Klein, O.D., and Sauvage, F.J.d. (2011). A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* 478, 255-259.
- Huels, D.J., Ridgway, R.A., Radulescu, S., Leushacke, M., Campbell, A.D., Biswas, S., Leedham, S., Serra, S., Chetty, R., Moreaux, G., *et al.* (2015). E-cadherin can limit the transforming properties of activating beta-catenin mutations. *EMBO J*, 1-13.
- Hui, L., Bakiri, L., Stepniak, E., and Wagner, E.F. (2007). p38alpha: a suppressor of cell proliferation and tumorigenesis. *Cell Cycle* 6, 2429-2433.
- Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., and Perucho, M. (1993). Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363, 558-561.
- Ipsaro, J.J., Huang, L., and Mondragon, A. (2009). Structures of the spectrin-ankyrin interaction binding domains. *Blood* 113, 5385-5393.
- Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A.R., Sansom, O.J., and Winton, D.J. (2004). Inducible cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of β -catenin. *Gastroenterology* 126, 1236-1246.
- Itasaki, N. (2003). Wise, a context-dependent activator and inhibitor of Wnt signalling. *Development* 130, 4295-4305.
- Itzkovitz, S., Lyubimova, A., Blat, I.C., Maynard, M., van Es, J., Lees, J., Jacks, T., Clevers, H., and van Oudenaarden, A. (2011). Single-molecule transcript counting of stem-cell markers in the mouse intestine. *Nature Cell Biology* 14, 106-114.
- Jadhav, U., Saxena, M., O'Neill, N.K., Saadatpour, A., Yuan, G.-C., Herbert, Z., Murata, K., and Shivdasani, R.A. (2017). Dynamic Reorganization of Chromatin Accessibility Signatures during Dedifferentiation of Secretory Precursors into Lgr5+ Intestinal Stem Cells. *Cell* 21, 65-77.

- Jaeger, E., Leedham, S., Lewis, A., Segditsas, S., Becker, M., Cuadrado, P.R., Davis, H., Kaur, K., Heinimann, K., Howarth, K., *et al.* (2012). Hereditary mixed polyposis syndrome is caused by a 40-kb upstream duplication that leads to increased and ectopic expression of the BMP antagonist GREM1. *Nature genetics* **44**, 699-703.
- Janda, C.Y., Waghray, D., Levin, A.M., Thomas, C., and Garcia, K.C. (2012). Structural Basis of Wnt Recognition by Frizzled. *Science* **337**, 59-64.
- Janssen, K.P., Alberici, P., Fsihi, H., Gaspar, C., Breukel // , C., Franken, P., Rosty, C., Abal, M., Marjou, F.E., Smits, R., *et al.* (2006). APC and Oncogenic KRAS Are Synergistic in Enhancing Wnt Signaling in Intestinal Tumor Formation and Progression. *Gastroenterology* **131**, 1096-1109.
- Jeong, W.-J., Yoon, J., Park, J.-C., Lee, S.-H., Lee, S.-H., Kaduwal, S., Kim, H., Yoon, J.-B., and Choi, K.-Y. (2012). Ras Stabilization Through Aberrant Activation of Wnt/b-Catenin Signaling Promotes Intestinal Tumorigenesis. *Science Signalling* **5**, 1-13.
- Ji, L., Jiang, B., Jiang, X., Charlat, O., Chen, A., Mickanin, C., Bauer, A., Xu, W., Yan, X., and Cong, F. (2017). The SIAH E3 ubiquitin ligases promote Wnt/ β -catenin signaling through mediating Wnt-induced Axin degradation. *Genes & Development* **31**, 1-12.
- Jian Wu, Yuchen Jiao, Marco Dal Molin, Anirban Maitra, Roeland F. de Wilde, Laura D. Wood, James R. Eshleman, Michael G. Goggins, Christopher L. Wolfgang, Marcia I. Canto, *et al.* (2011). Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 21188-21193.
- Jiang, X., Hao, H.-X., Growney, J.D., Woolfenden, S., Bottiglio, C., Ng, N., Lu, B., Hsieh, M.H., Bagdasarian, L., Meyer, R., *et al.* (2013). Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 12649-12654.
- Jin, L.H., Shao, Q.J., Luo, W., Ye, Z.Y., Li, Q., and Lin, S.C. (2003). Detection of point mutations of the Axin1 gene in colorectal cancers. *International journal of cancer Journal international du cancer* **107**, 696-699.
- Jones, S.E., and Jomary, C. (2002). Secreted Frizzled-related proteins: searching for relationships and patterns. *BioEssays* **24**, 811-820.
- Jose Saldanha, Juswinder Singh, and Mahadevan, D. (1998). Identification of a frizzled-like cysteine rich domain in the extracellular region of developmental receptor tyrosine kinases. *Protein Science* **7**, 1632–1635.
- Kabiri, Z., Greicius, G., Madan, B., Biechele, S., Zhong, Z., Zaribafzadeh, H., Edison, Aliyev, J., Wu, Y., Bunte, R., *et al.* (2014). Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts. *Development* **141**, 2206-2215.
- Kakugawa, S., Langton, P.F., Zebisch, M., Howell, S.A., Chang, T.H., Liu, Y., Feizi, T., Bineva, G., O'Reilly, N., Snijders, A.P., *et al.* (2015). Notum deacylates Wnt proteins to suppress signalling activity. *Nature* **519**, 187-192.

- Karaman, M.W., Herrgard, S., Treiber, D.K., Gallant, P., Atteridge, C.E., Campbell, B.T., Chan, K.W., Ciceri, P., Davis, M.I., Edeen, P.T., *et al.* (2008). A quantitative analysis of kinase inhibitor selectivity. *Nature biotechnology* 26, 127-132.
- Kelley, M.W. (2008). Leading Wnt down a PCP path: Cthrc1 acts as a coreceptor in the Wnt-PCP pathway. *Dev Cell* 15, 7-8.
- Kennell, J.A., O'Leary, E.E., Gummow, B.M., Hammer, G.D., and MacDougald, O.A. (2003). T-Cell Factor 4N (TCF-4N), a Novel Isoform of Mouse TCF-4, Synergizes with β -Catenin To Coactivate C/EBP and Steroidogenic Factor 1 Transcription Factors. *Molecular and cellular biology* 23, 5366-5375.
- Kikuchi, A., Yamamoto, H., Sato, A., and Matsumoto, S. (2011). New Insights into the Mechanism of Wnt Signaling Pathway Activation. *International Review of Cell and Molecular Biology* 291.
- Kim, H.A., Koo, B.K., Cho, J.H., Kim, Y.Y., Seong, J., Chang, H.J., Oh, Y.M., Stange, D.E., Park, J.G., Hwang, D., *et al.* (2012a). Notch1 counteracts WNT/beta-catenin signaling through chromatin modification in colorectal cancer. *J Clin Invest* 122, 3248-3259.
- Kim, K.-A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emtage, P., *et al.* (2005). Mitogenic Influence of Human R-Spondin1 on the Intestinal Epithelium. *Science* 309, 1256-1259.
- Kim, K.H., Lee, T.R., and Cho, E.G. (2017). SH3BP4, a novel pigmentation gene, is inversely regulated by miR-125b and MITF. *Experimental Molecular Medicine* 49, 1-7.
- Kim, S., Xu, X., Hecht, A., and Boyer, T.G. (2006). Mediator is a transducer of Wnt/beta-catenin signaling. *The Journal of biological chemistry* 281, 14066-14075.
- Kim, Y.M., and Kim, D.H. (2013). dRAGging amino acid-mTORC1 signaling by SH3BP4. *Molecules and cells* 35, 1-6.
- Kim, Y.M., Stone, M., Hwang, T.H., Kim, Y.G., Dunlevy, J.R., Griffin, T.J., and Kim, D.H. (2012b). SH3BP4 is a negative regulator of amino acid-Rag GTPase-mTORC1 signaling. *Molecular cell* 46, 833-846.
- Kima, T.-H., Escudero, S., and Shivdasani, R.A. (2012). Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells. *Proceedings of the National Academy of Sciences of the United States of America* 109, 3932–3937.
- Klaus Giese, Amsterdam, A., and Grosschedl, R. (1991). DNA-binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1. *Genes & Development* 5, 2567-2578.
- Kohler, E.M., Chandra, S.H., Behrens, J., and Schneikert, J. (2009). Beta-catenin degradation mediated by the CID domain of APC provides a model for the selection of APC mutations in colorectal, desmoid and duodenal tumours. *Hum Mol Genet* 18, 213-226.

- Koinuma, K., Yamashita, Y., Liu, W., Hatanaka, H., Kurashina, K., Wada, T., Takada, S., Kaneda, R., Choi, Y.L., Fujiwara, S.I., *et al.* (2006). Epigenetic silencing of AXIN2 in colorectal carcinoma with microsatellite instability. *Oncogene* 25, 139-146.
- Kokoszyńska, K., Kryński, J., Rychlewski, L., and Wyrwicz, L.S. (2009). Unexpected domain composition of MACC1 links MET signaling and apoptosis. *Acta Biochimica Polonia* 56 317–323.
- Komekado, H., Yamamoto, H., Chiba, T., and Kikuchi1, A. (2007). Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes to Cells* 12, 521-534.
- Koo, B.K., Spit, M., Jordens, I., Low, T.Y., Stange, D.E., van de Wetering, M., van Es, J.H., Mohammed, S., Heck, A.J., Maurice, M.M., *et al.* (2012). Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature* 488, 665-669.
- Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P.J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nature genetics* 19, 379-383.
- Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 275, 1784-1787.
- Korkut, C., Ataman, B., Ramachandran, P., Ashley, J., Barria, R., Gherbesi, N., and Budnik, V. (2009). Trans-synaptic transmission of vesicular Wnt signals through Evi/Wntless. *Cell* 139, 393-404.
- Kormish, J.D., Sinner, D., and Zorn, A.M. (2010). Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease. *Developmental dynamics : an official publication of the American Association of Anatomists* 239, 56-68.
- Kranz, A., Fu, J., Duerschke, K., Weidlich, S., Naumann, R., Stewart, A.F., and Anastassiadis, K. (2010). An improved Flp deleter mouse in C57Bl/6 based on Flpo recombinase. *Genesis* 48, 512-520.
- Krausova, M., and Korinek, V. (2013). Wnt signaling in adult intestinal stem cells and cancer. *Cellular signalling* 26, 570-579.
- Krupnika, V.E., Sharpb, J.D., Jiangc, C., Robisond, K., Chickeringc, T.W., Amaravadic, L., Brown, D.E., Guyot, D., Maysc, G., Leibyc, K., *et al.* (1999). Functional and structural diversity of the human Dickkopf gene family. *Gene* 238, 301-313.
- Kurayoshi, M., Yamamoto, H., Izumi, S., and Kikuchi, A. (2007). Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *The Biochemical journal* 402, 515-523.
- Kwon, C., Cheng, P., King, I.N., Andersen, P., Shenje, L., Nigam, V., and Srivastava, D. (2011). Notch post-translationally regulates β -catenin protein in stem and progenitor cells. *Nat Cell Biol* 13, 1244 -1251.

- Lammi, L., Arte, S., Somer, M., Jarvinen, H., Lahermo, P., Thesleff, I., Pirinen, S., and Nieminen, P. (2004). Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. *American journal of human genetics* 74, 1043-1050.
- Lau, W.d., Peng, W.C., Gros, P., and Clevers, H. (2014). The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. *Genes & Development* 28, 305–316.
- Li, V.S., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J., Maurice, M.M., Mahmoudi, T., *et al.* (2012). Wnt signaling through inhibition of beta-catenin degradation in an intact Axin1 complex. *Cell* 149, 1245-1256.
- Li-KuoSu, Kinzler, K., BertVogelstein, Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., and Dove, W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 256, 668-670.
- Liao, G., Tao, Q., Kofron, M., Chen, J.-S., Schloemer, A., Davis, R.J., Hsieh, J.-C., Wylie, C., Heasman, J., and Kuan, C.-Y. (2006). Jun NH2-terminal kinase (JNK) prevents nuclear β -catenin accumulation and regulates axis formation in *Xenopus* embryos. *Proceedings of the National Academy of Sciences of the United States of America* 103, 16313-16318.
- Lilien, J., and Balsamo, J. (2005). The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. *Curr Opin Cell Biol* 17, 459-465.
- Lim, X., Tan, S.H., Koh, W.L., Chau, R.M., Yan, K.S., Kuo, C.J., van Amerongen, R., Klein, A.M., and Nusse, R. (2013). Interfollicular epidermal stem cells self-renew via autocrine Wnt signaling. *Science* 342, 1226-1230.
- Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., and He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108, 837-847.
- Liu, C.C., Kanekiyo, T., Roth, B., and Bu, G. (2014a). Tyrosine-based signal mediates LRP6 receptor endocytosis and desensitization of Wnt/beta-catenin pathway signaling. *The Journal of biological chemistry* 289, 27562-27570.
- Liu, G., Bafico, A., Harris, V.K., and Aaronson, S.A. (2003). A Novel Mechanism for Wnt Activation of Canonical Signaling through the LRP6 Receptor. *Molecular and cellular biology* 23, 5825-5835.
- Liu, J., McClelland, M., Stawiski, E.W., Gnad, F., Mayba, O., Haverty, P.M., Durinck, S., Chen, Y.J., Klijn, C., Jhunjhunwala, S., *et al.* (2014b). Integrated exome and transcriptome sequencing reveals ZAK isoform usage in gastric cancer. *Nature communications* 5, 1-8.
- Liu, T.C., Huang, C.J., Chu, Y.C., Wei, C.C., Chou, C.C., Chou, M.Y., Chou, C.K., and Yang, J.J. (2000a). Cloning and expression of ZAK, a mixed lineage kinase-like protein containing a leucine-zipper and a sterile-alpha motif. *Biochem Biophys Res Commun* 274, 811-816.
- Liu, W., Dong, X., Mai, M., Seelan, R.S., Taniguchi, K., Krishnadath, K.K., Halling, K.C., Cunningham, J.M., Boardman, L.A., Qian, C., *et al.* (2000b). Mutations in AXIN2 cause

colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. *Nature genetics* 26, 146-147.

Liu, X., Rubin, J.S., and Kimmel, A.R. (2005). Rapid, Wnt-Induced Changes in GSK3 β Associations that Regulate b-Catenin Stabilization Are Mediated by G α Proteins. *Current Biology* 15, 1989–1997.

Llosa, N.J., Cruise, M., Tam, A., Wicks, E.C., Hechenbleikner, E.M., Taube, J.M., Blosser, R.L., Fan, H., Wang, H., Lubber, B.S., *et al.* (2015). The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov* 5, 43-51.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20, 781-810.

Lopez-Garcia, C., Klein, A.M., Simons, B.D., and Winton, D.J. (2010). Intestinal Stem Cell Replacement Follows a Pattern of Neutral Drift. *Science* 330.

Loregger, A., Grandl, M., Mejías-Luque, R., Allgäuer, M., Degenhart, K., Haselmann, V., Oikonomou, C., Hatzis, P., Janssen, K.-P., Nitsche, U., *et al.* (2015). The E3 ligase RNF43 inhibits Wnt signaling downstream of mutated b-catenin by sequestering TCF4 to the nuclear membrane. *Science Signaling* 8, 1-13.

Love, J.J., Li, X., Case, D.A., Giese, K., Crosschedl, R., and Wright, P.E. (1995). Structural basis for DNA bending by the architectural transcription factor LEF-1. *Nature* 376, 791-795.

Lu, W., Yamamoto, V., Ortega, B., and Baltimore, D. (2004). Mammalian Ryk Is a Wnt Coreceptor Required for Stimulation of Neurite Outgrowth. *Cell* 119, 97-108.

Luke, G.N., Castro, L.F.C., McLay, K., Bird, C., Coulson, A., and Holland, P.W.H. (2003). Dispersal of NK homeobox gene clusters in amphioxus and humans. *Proceedings of the National Academy of Sciences of the United States of America* 100, 5292-5295.

Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W., *et al.* (2002). Negative Feedback Loop of Wnt Signaling through Upregulation of Conductin/Axin2 in Colorectal and Liver Tumors. *Molecular and cellular biology* 22, 1184-1193.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17, 9-26.

Major, M.B., Camp, N.D., Berndt, J.D., Yi, X., Goldenberg, S.J., Hubbert, C., Biechele, T.L., Gingras, A.C., Zheng, N., Maccoss, M.J., *et al.* (2007). Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling. *Science* 316, 1043-1046.

Mao, B., and Niehrs, C. (2003). Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. *Gene* 302, 179-183.

Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B.M., Delius, H., Hoppe, D., Stanek, P., Walter, C., *et al.* (2002a). Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature* 417, 664-667.

Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B.M., Delius, H., Hoppe, D., Stannek, P., Walter, C., *et al.* (2002b). Kremen proteins are Dickkopf receptors that regulate Wnt/ β -catenin signalling. *Nature* **417**, 664-667.

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (2001). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* **411**, 321-325.

Marvin, M.L., Mazzoni, S.M., Herron, C.M., Edwards, S., Gruber, S.B., and Petty, E.M. (2011). AXIN2-associated autosomal dominant ectodermal dysplasia and neoplastic syndrome. *American journal of medical genetics Part A* **155A**, 898-902.

Massagué, J., Seoane, J., and Wotton, D. (2005). Smad transcription factors. *Genes & Development* **19**, 2783-2810.

Mayer, B.J. (2001). SH3 domains: complexity in moderation. *Signal Transduction and Cellular Organization* **114**, 1253-1263.

Meran, L., Baulies, A., and Li, V.S.W. (2017). Intestinal Stem Cell Niche: The Extracellular Matrix and Cellular Components. *Stem Cells Int* **2017**, 7970385.

Merlos-Suarez, A., Barriga, F.M., Jung, P., Iglesias, M., Cespedes, M.V., Rossell, D., Sevillano, M., Hernando-Momblona, X., da Silva-Diz, V., Munoz, P., *et al.* (2011). The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. *Cell stem cell* **8**, 511-524.

Metcalfe, C., Kljavin, N.M., Ybarra, R., and de Sauvage, F.J. (2014). Lgr5+ stem cells are indispensable for radiation-induced intestinal regeneration. *Cell stem cell* **14**, 149-159.

Miller, H., Zhang, J., KuoLee, R., Patel, G.B., and Chen, W. (2007). Intestinal M cells: The fallible sentinels? *World Journal of Gastroenterology* **13**, 1477-1486.

Mills, J.C., and Gordon, J.I. (2001). The intestinal stem cell niche: There grows the neighborhood. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 12334-12336.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86**, 391-399.

Montgomery, R.K., Carlone, D.L., Richmond, C.A., Farilla, L., Kranendonk, M.E.G., Henderson, D.E., Baffour-Awuaha, N.Y., Ambruzsc, D.M., Foglic, L.K., Algraa, S., *et al.* (2011). Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 179-184.

Moon, R.T., Campbell, R.M., Christian, J.L., McGrew, L.L., Shih, J., and Fraser, S. (1993). Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*. *Development* **119**, 97-111.

Moran, A.E., Hunt, D.H., Javid, S.H., Redston, M., Carothers, A.M., and Bertagnolli, M.M. (2004). Apc deficiency is associated with increased Egfr activity in the intestinal

- enterocytes and adenomas of C57BL/6J-Min/+ mice. *The Journal of biological chemistry* 279, 43261-43272.
- Mori-Akiyama, Y., Born, M.v.d., Es, J.H.v., Hamilton, S.R., Adams, H.P., Zhang, J., Clevers, H., and Crombrugghe, B.d. (2007). SOX9 Is Required for the Differentiation of Paneth Cells in the Intestinal Epithelium. *Gastroenterology* 133, 539–546.
- Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275, 1787-1790.
- Moser, A.R., Pitot, H.C., and Dove, W.F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 247, 322-324.
- Mosimann, C., Hausmann, G., and Basler, K. (2006). Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo. *Cell* 125, 327-341.
- Munoz, J., Stange, D.E., Schepers, A.G., van de Wetering, M., Koo, B.K., Itzkovitz, S., Volckmann, R., Kung, K.S., Koster, J., Radulescu, S., *et al.* (2012). The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. *EMBO J* 31, 3079-3091.
- Murakami, T., Mitomi, H., Saito, T., Takahashi, M., Sakamoto, N., Fukui, N., Yao, T., and Watanabe, S. (2014). Distinct WNT/beta-catenin signaling activation in the serrated neoplasia pathway and the adenoma-carcinoma sequence of the colorectum. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc.*
- Nagaraj, R., and Banerjee, U. (2009). Regulation of Notch and Wingless signalling by phyllopod, a transcriptional target of the EGFR pathway. *EMBO Journal* 28, 337-346.
- Nagase, H., and Nakamura, Y. (1993). Mutations of the APC (adenomatous polyposis coli) gene. *Human mutation* 2, 425-434.
- Nakanishi, Y., Seno, H., Fukuoka, A., Ueo, T., Yamaga, Y., Maruno, T., Nakanishi, N., Kanda, K., Komekado, H., Kawada, M., *et al.* (2013). Dclk1 distinguishes between tumor and normal stem cells in the intestine. *Nature genetics* 45, 98-103.
- Nihalani, D., Merritt, S., and Holzman, L.B. (2000). Identification of Structural and Functional Domains in Mixed Lineage Kinase Dual Leucine Zipper-bearing Kinase Required for Complex Formation and Stress-activated Protein Kinase Activation. *The Journal of biological chemistry* 10, 7273-7279.
- Nishida, E., and Gotoh, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends in biochemical sciences* 18, 128-131.
- Noah, T.K., Donahue, B., and Shroyer, N.F. (2011). Intestinal development and differentiation. *Experimental cell research* 317, 2702-2710.
- Novellasedemunt, L., Foglizzo, V., Cuadrado, L., Antas, P., Kucharska, A., Encheva, V., Snijders, A.P., and Li, V.S.W. (2017). USP7 Is a Tumor-Specific WNT Activator for APC-

- Mutated Colorectal Cancer by Mediating beta-Catenin Deubiquitination. *Cell Rep* 21, 612-627.
- Nusse, R., Brown, A., Papkoff, J., Scambler, P., Shackleford, G., McMahon, A., Moon, R., and Varmus, H. (1991). A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell* 64, 231-232.
- Nusse, R., and Clevers, H. (2017). Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* 169, 985-999.
- Nusse, R., and Varmus, H. (2012). Three decades of Wnts: a personal perspective on how a scientific field developed. *EMBO J* 31, 2670-2684.
- Nusse, R., and Varmus, H.E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31, 99-109.
- O'Brien, C.A., Pollett, A., Gallinger, S., and Dick, J.E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 445, 106-110.
- Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., Koshida, I., Suzuki, K., Yamada, G., Schwabe, G.C., *et al.* (2003). The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes to Cells* 8, 645-654.
- Ong, C.K., Subimerb, C., Pairojkul, C., Wongkham, S., Cutcutache, I., Yu, W., McPherson, J.R., Allen, G.E., Ng, C.C., Wong, B.H., *et al.* (2012). Exome sequencing of liver fluke-associated cholangiocarcinoma. *Nature genetics* 44, 690-693.
- Ordonez-Moran, P., Larriba, M.J., Palmer, H.G., Valero, R.A., Barbachano, A., Dunach, M., de Herreros, A.G., Villalobos, C., Berciano, M.T., Lafarga, M., *et al.* (2008). RhoA-ROCK and p38MAPK-MSK1 mediate vitamin D effects on gene expression, phenotype, and Wnt pathway in colon cancer cells. *J Cell Biol* 183, 697-710.
- Orford, K., Crockett, C., Jensen, J.P., Weissman, A.M., and Byers, S.W. (1997). Serine Phosphorylation- regulated Ubiquitination and Degradation of Beta-Catenin. *The Journal of biological chemistry* 272, 24735-24738.
- Peignon, G., Durand, A., Cacheux, W., Ayrault, O., Terris, B., Laurent-Puig, P., Shroyer, N.F., Seuringen, I.V., Honjo, T., Perret, C., *et al.* (2011). Complex interplay between b-catenin signalling and Notch effectors in intestinal tumorigenesis. *Gut* 60, 166-176.
- Petherick, K.J., Williams, A.C., Lane, J.D., Ordonez-Moran, P., Huelsken, J., Collard, T.J., Smartt, H.J., Batson, J., Malik, K., Paraskeva, C., *et al.* (2013). Autolysosomal beta-catenin degradation regulates Wnt-autophagy-p62 crosstalk. *EMBO J* 32, 1903-1916.
- Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J., and Skarnes, W.C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407, 535-538.
- Port, F., Kuster, M., Herr, P., Furger, E., Banziger, C., Hausmann, G., and Basler, K. (2008). Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nat Cell Biol* 10, 178-185.

- Potten, C.S. (1977). Extreme sensitivity of some intestinal crypt cells to X and gamma-radiation. *Nature*, 518-521.
- Potten, C.S., and Grant, H.K. (1998). The relationship between ionizing radiation-induced apoptosis and stem cells in the small and large intestine. *British Journal of Cancer* 78, 993-1003.
- Powell, A.E., Wang, Y., Li, Y., Poulin, E.J., Means, A.L., Washington, M.K., Higginbotham, J.N., Juchheim, A., Prasad, N., Levy, S.E., *et al.* (2012). The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. *Cell* 149, 146-158.
- Powell, D.W., Mifflin, R.C., Valentich, J.D., Crowe, S.E., Saada, J.I., and West, A.B. (1999). Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *American Journal of Physiology - Cell Physiology* 277.
- Qi, J., Zhu, Y.Q., Luo, J., and Tao, W.H. (2006). Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor. *World journal of gastroenterology : WJG* 12, 7113-7117.
- Qi, Z., Li, Y., Zhao, B., Xu, C., Liu, Y., Li, H., Zhang, B., Wang, X., Yang, X., Xie, W., *et al.* (2017). BMP restricts stemness of intestinal Lgr5+ stem cells by directly suppressing their signature genes. *Nature communications* 8, 13824.
- Rahman, M.S., Akhtar, N., Jamil, H.M., Banik, R.S., and Asaduzzaman, S.M. (2015). TGF-beta/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. *Bone Research* 3, 15005.
- Rajagopalan, H., Bardelli, A., Lengauer, C., Kinzler, K.W., Vogelstein, B., and Velculescu, V.E. (2002). Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* 418.
- Ramesh A. Bhat, Barbara Stauffer, Barry S. Komm, and Bodine, P.V.N. (2007). Structure-Function analysis of secreted frizzled-related protein-1 for its Wnt antagonist function. *The Journal of biological chemistry* 102, 1519-1528.
- Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature protocols* 8, 2281-2308.
- Rawson, J.B., Manno, M., Mrkonjic, M., Daftary, D., Dicks, E., Buchanan, D.D., Younghusband, H.B., Parfrey, P.S., Young, J.P., Pollett, A., *et al.* (2011). Promoter methylation of Wnt antagonists DKK1 and SFRP1 is associated with opposing tumor subtypes in two large populations of colorectal cancer patients. *Carcinogenesis* 32, 741-747.
- Reed, J.C., Doctor, K.S., and Godzik, A. (2004). The Domains of Apoptosis: A Genomics Perspective. *Science's STKE* 2004.
- Rey, C., Faustin, B., Mahouche, I., Ruggieri, R., Brulard, C., Ichas, F., Soubeyran, I., Lartigue, L., and Giorgi, F.D. (2015). The MAP3K ZAK, a novel modulator of ERK-dependent migration, is upregulated in colorectal cancer. *Oncogene*, 1-11.

- Ricci-Vitiani, L., Lombardi, D.G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., and De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature* 445, 111-115.
- Rios-Esteves, J., Haugen, B., and Resh, M.D. (2014). Identification of key residues and regions important for porcupine-mediated Wnt acylation. *The Journal of biological chemistry* 289, 17009-17019.
- Rios-Esteves, J., and Resh, M.D. (2013). Stearoyl CoA desaturase is required to produce active, lipid-modified Wnt proteins. *Cell Rep* 4, 1072-1081.
- Rivera, B., Perea, J., Sanchez, E., Villapun, M., Sanchez-Tome, E., Mercadillo, F., Robledo, M., Benitez, J., and Urioste, M. (2014). A novel AXIN2 germline variant associated with attenuated FAP without signs of oligodontia or ectodermal dysplasia. *European journal of human genetics : EJHG* 22, 423-426.
- Robertis, M.D., Arigoni, M., Loiacono, L., Riccardo, F., Calogero, R.A., Feodorova, Y., Tashkova, D., Belovejdov, V., Sara, V., Cavallo, F., *et al.* (2015). Novel insights into Notum and glypicans regulation in colorectal cancer. *Oncotarget* 6, 41237–41257.
- Roberts, D.M., Pronobis, M.I., Poulton, J.S., Waldmann, J.D., Stephenson, E.M., Hanna, S., and Peifer, M. (2011). Deconstructing the β catenin destruction complex: mechanistic roles for the tumor suppressor APC in regulating Wnt signaling. *Molecular Biology of the Cell* 22, 1845-1863.
- Robinson, M.J., and Cobb, M.H. (1997). Mitogen-activated protein kinase pathways. *Current opinion in cell biology* 9, 180-186.
- Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., Wetering, M.v.d., Destree, O., and Clevers, H. (1998). The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 395, 608-612.
- Rosin-Arbesfeld, R., Townsley, F., and Bienz, M. (2000). The APC tumour suppressor has a nuclear export function. *Nature* 406, 1009-1012.
- Roth, S., Franken, P., Sacchetti, A., Kremer, A., Anderson, K., Sansom, O., and Fodde, R. (2012). Paneth cells in intestinal homeostasis and tissue injury. *PLoS One* 7, e38965.
- Rothenberg, M.E., Nusse, Y., Kalisky, T., Lee, J.J., Dalerba, P., Scheeren, F., Lobo, N., Kulkarni, S., Sim, S., Qian, D., *et al.* (2012). Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+) stem cells in mice. *Gastroenterology* 142, 1195-1205.
- Ryland, G.L., Hunter, S.M., Doyle, M.A., Rowley, S.M., Christie, M., Allan, P.E., Bowtell, D.D., Australian Ovarian Cancer Study, G., Gorringe, K.L., and Campbell, I.G. (2013). RNF43 is a tumour suppressor gene mutated in mucinous tumours of the ovary. *J Pathol* 229, 469-476.
- Saha, S., Aranda, E., Hayakawa, Y., Bhanja, P., Atay, S., Brodin, N.P., Li, J., Asfaha, S., Liu, L., Tailor, Y., *et al.* (2016). Macrophage-derived extracellular vesicle-packaged WNTs rescue intestinal stem cells and enhance survival after radiation injury. *Nature communications* 7, 13096.

- Sailaja, B.S., He, X.C., and Li, L. (2016). The regulatory niche of intestinal stem cells. *J Physiol* 594, 4827-4836.
- Salahshor, S., and Woodgett, J.R. (2005). The links between axin and carcinogenesis. *Journal of clinical pathology* 58, 225-236.
- Salic, A., Lee, E., Mayer, L., and Kirschner, M.W. (2000). Control of β -Catenin Stability: Reconstitution of the Cytoplasmic Steps of the Wnt Pathway in *Xenopus* Egg Extracts. *Molecular cell* 5, 523-532.
- Sancho, R., Cremona, C.A., and Behrens, A. (2015). Stem cell and progenitor fate in the mammalian intestine: Notch and lateral inhibition in homeostasis and disease. *EMBO Rep* 16, 571-581.
- Sangiorgi, E., and Capecchi, M.R. (2008). *Bmi1* is expressed in vivo in intestinal stem cells. *Nature genetics* 40.
- Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., *et al.* (2004). Loss of *Apc* in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes & Development* 18, 1385–1390.
- Sasaki, N., Sachs, N., Wiebrands, K., Ellenbroek, S.I., Fumagalli, A., Lyubimova, A., Begthel, H., van den Born, M., van Es, J.H., Karthaus, W.R., *et al.* (2016). *Reg4+* deep crypt secretory cells function as epithelial niche for *Lgr5+* stem cells in colon. *Proceedings of the National Academy of Sciences of the United States of America* 113, 5399-5407.
- Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H. (2011). Paneth cells constitute the niche for *Lgr5* stem cells in intestinal crypts. *Nature* 469, 415-418.
- Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., *et al.* (2009). Single *Lgr5* stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459, 262-265.
- Schepers, A.G., Vries, R., van den Born, M., van de Wetering, M., and Clevers, H. (2011). *Lgr5* intestinal stem cells have high telomerase activity and randomly segregate their chromosomes. *EMBO J* 30, 1104-1109.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676-682.
- Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P.J., Kikuchi, A., Higuchi, Y., and Bienz, M. (2007). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. *Nature Structural Molecular Biology* 14, 484-492.
- Schwitalla, S., Fingerle, A.A., Cammareri, P., Nebelsiek, T., Goktuna, S.I., Ziegler, P.K., Canli, O., Heijmans, J., Huels, D.J., Moreaux, G., *et al.* (2013). Intestinal Tumorigenesis Initiated by Dedifferentiation and Acquisition of Stem-Cell-like Properties. *Cell* 152, 25-38.

- Sekiya, T., and Zaret, K.S. (2007). Repression by Groucho/TLE/Grg proteins: genomic site recruitment generates compacted chromatin in vitro and impairs activator binding in vivo. *Molecular cell* 28, 291-303.
- Semënov, M., Tamai, K., and He, X. (2005). SOST Is a Ligand for LRP5/LRP6 and a Wnt Signaling Inhibitor. *The Journal of biological chemistry* 280, 26770-26775.
- Seshagiri, S., Stawiski, E.W., Durinck, S., Modrusan, Z., Storm, E.E., Conboy, C.B., Chaudhuri, S., Guan, Y., Janakiraman, V., Jaiswal, B.S., *et al.* (2012). Recurrent R-spondin fusions in colon cancer. *Nature* 488, 660-664.
- Sheldahl, L.C., Slusarski, D.C., Pandur, P., Miller, J.R., Kuhl, M., and Moon, R.T. (2003). Dishevelled activates Ca²⁺ flux, PKC, and CamKII in vertebrate embryos. *J Cell Biol* 161, 769-777.
- Shimizu, Y., Ikeda, S., Fujimori, M., Kodama, S., Nakahara, M., Okajima, M., and Asahara, T. (2002). Frequent alterations in the Wnt signaling pathway in colorectal cancer with microsatellite instability. *Genes, chromosomes & cancer* 33, 73-81.
- Shimomura, Y., Agalliu, D., Vonica, A., Luria, V., Wajid, M., Baumer, A., Belli, S., Petukhova, L., Schinzel, A., Brivanlou, A.H., *et al.* (2010). APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. *Nature* 464, 1043-1047.
- Siegel, R.L., Miller, K.D., and Jemal, A. (2017). Cancer Statistics, 2017. *CA Cancer J Clin* 67, 7-30.
- Silva, A.L., Dawson, S.N., Arends, M.J., Guttula, K., Hall, N., Cameron, E.A., Huang, T.H., Brenton, J.D., Tavare, S., Bienz, M., *et al.* (2014). Boosting Wnt activity during colorectal cancer progression through selective hypermethylation of Wnt signaling antagonists. *BMC cancer* 14.
- Simons, M., Gault, W.J., Gotthardt, D., Rohatgi, R., Klein, T.J., Shao, Y., Lee, H.J., Wu, A.L., Fang, Y., Satlin, L.M., *et al.* (2009). Electrochemical cues regulate assembly of the Frizzled/Dishevelled complex at the plasma membrane during planar epithelial polarization. *Nat Cell Biol* 11, 286-294.
- Sjoberg, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N., *et al.* (2006). The consensus coding sequences of human breast and colorectal cancers. *Science* 314, 268-274.
- Snippert, H.J., Haegebarth, A., Kasper, M., Jaks, V., Es, J.H.v., Barker, N., Wetering, M.v.d., Born, M.v.d., Begthel, H., Vries, R.G., *et al.* (2010a). Lgr6 Marks Stem Cells in the Hair Follicle That Generate All Cell Lineages of the Skin. *Science* 327, 1385-1389.
- Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D., *et al.* (2010b). Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 143, 134-144.
- Snippert, H.J., van Es, J.H., van den Born, M., Begthel, H., Stange, D.E., Barker, N., and Clevers, H. (2009). Prolimin-1/CD133 marks stem cells and early progenitors in mouse small intestine. *Gastroenterology* 136, 2187-2194.

- Spielmann, M., Kakar, N., Tayebi, N., Leettola, C., Nurnberg, G., Sowada, N., Lupianez, D.G., Harabula, I., Flottmann, R., Horn, D., *et al.* (2016). Exome sequencing and CRISPR/Cas genome editing identify mutations of ZAK as a cause of limb defects in humans and mice. *Genome Res* 26, 183-191.
- Spink, K.E., G.Fridman, S., and I.Weis, W. (2001). Molecular mechanisms of β -catenin recognition by adenomatous polyposis coli revealed by the structure of an APC- β -catenin complex. *The EMBO journal* 20, 6203-6212.
- Stapleton, D., Balan, I., Pawson, T., and Sicheri, F. (1999). The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization. *Nature Structural & Molecular Biology* 6, 44-49.
- Stark, K., Vainio, S., Vassileva, G., and McMahon, A.P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 372, 679 - 683
- Stein, U., Dahlmann, M., and Walther, W. (2010). MACC1 - more than metastasis? Facts and predictions about a novel gene. *Journal of molecular medicine* 88, 11-18.
- Stein, U., Walther, W., Arlt, F., Schwabe, H., Smith, J., Fichtner, I., Birchmeier, W., and Schlag, P.M. (2009). MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis. *Nat Med* 15, 59-67.
- Sue Ng, S., Mahmoudi, T., Li, V.S., Hatzis, P., Boersema, P.J., Mohammed, S., Heck, A.J., and Clevers, H. (2010). MAP3K1 functionally interacts with Axin1 in the canonical Wnt signalling pathway. *Biological chemistry* 391, 171-180.
- Surmann-Schmitt, C., Widmann, N., Dietz, U., Saeger, B., Eitzinger, N., Nakamura, Y., Rattel, M., Latham, R., Hartmann, C., von der Mark, H., *et al.* (2009). Wif-1 is expressed at cartilage-mesenchyme interfaces and impedes Wnt3a-mediated inhibition of chondrogenesis. *J Cell Sci* 122, 3627-3637.
- Suzuki, H., Watkins, D.N., Jair, K.W., Schuebel, K.E., Markowitz, S.D., Chen, W.D., Pretlow, T.P., Yang, B., Akiyama, Y., Van Engeland, M., *et al.* (2004). Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nature genetics* 36, 417-422.
- Suzuki, T., Kusakabe, M., Nakayama, K., and Nishida, E. (2012). The protein kinase MLTK regulates chondrogenesis by inducing the transcription factor Sox6. *Development* 139, 2988-2998.
- Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., and Takada, S. (2006). Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Dev Cell* 11, 791-801.
- Takahashi, N., Yamaguchi, K., Ikenoue, T., Fujii, T., and Furukawa, Y. (2014). Identification of two Wnt-responsive elements in the intron of RING finger protein 43 (RNF43) gene. *PLoS One* 9, 1-9.
- Takeda, N., Jain, R., LeBoeuf, M.R., Wang, Q., Lu, M.M., and Epstein, J.A. (2011). Interconversion Between Intestinal Stem Cell Populations in Distinct Niches. *Science* 334, 1420-1424.

- Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J.-P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* 407, 530-535.
- Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T., and Honjo, T. (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-Jk/Su(H). *Current Biology* 5, 1416-1423.
- Taniguchi, H., Yamamoto, H., Hirata, T., Miyamoto, N., Oki, M., Noshio, K., Adachi, Y., Endo, T., Imai, K., and Shinomura, Y. (2005). Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* 24, 7946-7952.
- Tanneberger, K., Pfister, A.S., Brauburger, K., Schneikert, J., Hadjihannas, M.V., Kriz, V., Schulte, G., Bryja, V., and Behrens, J. (2011). Amer1/WTX couples Wnt-induced formation of PtdIns(4,5)P₂ to LRP6 phosphorylation. *EMBO J* 30, 1433-1443.
- Tauriello, D.V.F., Jordens, I., Kirchner, K., Slootstra, J.W., Kruitwagen, T., Bouwman, B.A.M., Noutsou, M., Rüdiger, S.G.D., Schwamborn, K., Schambony, A., *et al.* (2012). Wnt/ β -catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proceedings of the National Academy of Sciences of the United States of America* 109, 812-820.
- TCGA (2012). Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 487, 330-337.
- Tetteh, P.W., Basak, O., Farin, H.F., Wiebrands, K., Kretzschmar, K., Begthel, H., van den Born, M., Korving, J., de Sauvage, F., van Es, J.H., *et al.* (2016). Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters. *Cell stem cell* 18, 203-213.
- Thibodeau, S.N., Bren, G., and Schaid, D. (1993). Microsatellite instability in cancer of the proximal colon. *Science* 260, 816-819.
- Thornton, T.M., Pedraza-Alva, G., Deng, B., Wood, C.D., Aronshtam, A., Clements, J.L., Sabio, G., Davis, R.J., Matthews, D.E., Doble, B., *et al.* (2008). Phosphorylation by p38 MAPK as an alternative pathway for GSK3 β inactivation. *Science* 320, 667-670.
- Thorstensen, L., Lind, G.E., Lovig, T., Diep, C.B., Meling, G.I., Rognum, T.O., and Lothe, R.A. (2005). Genetic and epigenetic changes of components affecting the WNT pathway in colorectal carcinomas stratified by microsatellite instability. *Neoplasia* 7, 99-108.
- Tienen, L.M.v., Mieszczynek, J., Fiedler, M., Rutherford, T.J., and Bienz, M. (2017). Constitutive scaffolding of multiple Wnt enhanceosome components by Legless/BCL9. *eLife* 6.
- Topol, L., Chen, W., Song, H., Day, T.F., and Yang, Y. (2009). Sox9 inhibits Wnt signaling by promoting β -catenin phosphorylation in the nucleus. *The Journal of biological chemistry* 284, 3323-3333.
- Torisu, Y., Watanabe, A., Nonaka, A., Midorikawa, Y., Makuuchi, M., Shimamura, T., Sugimura, H., Niida, A., Akiyama, T., Iwanari, H., *et al.* (2008). Human homolog of NOTUM, overexpressed in hepatocellular carcinoma, is regulated transcriptionally by β -catenin/TCF. *Cancer science* 99, 1139-1146.

- Tosoni, D., Puri, C., Confalonieri, S., Salcini, A.E., De Camilli, P., Tacchetti, C., and Di Fiore, P.P. (2005). TTP specifically regulates the internalization of the transferrin receptor. *Cell* 123, 875-888.
- Tree, D.R.P., Shulman, J.M., Rousset, R., Scott, M.P., Gubb, D., and Axelrod, J.D. (2002). Prickle Mediates Feedback Amplification to Generate Asymmetric Planar Cell Polarity Signaling. *Cell* 109, 371-381.
- Umbhauer, M., Djiane, A., Goisset, C., Penzo-Méndez, A., Riou, J.F., Boucaut, J.C., and Shi, D.L. (2000). The C-terminal cytoplasmic Lys-Thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/ β -catenin signalling. *The EMBO journal* 19, 4944-4954.
- Urra, H., Dufey, E., Avril, T., Chevet, E., and Hetz, C. (2016). Endoplasmic Reticulum Stress and the Hallmarks of Cancer. *Trends in Cancer* 2, 252-262.
- van Amerongen, R. (2012). Alternative Wnt pathways and receptors. *Cold Spring Harbor perspectives in biology* 4, 1-18.
- van der Flier, L.G., Haegebarth, A., Stange, D.E., van de Wetering, M., and Clevers, H. (2009a). OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* 137, 15-17.
- Van der Flier, L.G., Sabates-Bellver, J., Oving, I., Haegebarth, A., De Palo, M., Anti, M., Van Gijn, M.E., Suijkerbuijk, S., Van de Wetering, M., Marra, G., *et al.* (2007). The Intestinal Wnt/TCF Signature. *Gastroenterology* 132, 628-632.
- van der Flier, L.G., van Gijn, M.E., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D.E., Begthel, H., van den Born, M., Guryev, V., Oving, I., *et al.* (2009b). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* 136, 903-912.
- van Es, J.H., Haegebarth, A., Kujala, P., Itzkovitz, S., Koo, B.K., Boj, S.F., Korving, J., van den Born, M., van Oudenaarden, A., Robine, S., *et al.* (2012a). A critical role for the Wnt effector Tcf4 in adult intestinal homeostatic self-renewal. *Molecular and cellular biology* 32, 1918-1927.
- van Es, J.H., Sato, T., van de Wetering, M., Lyubimova, A., Nee, A.N., Gregorieff, A., Sasaki, N., Zeinstra, L., van den Born, M., Korving, J., *et al.* (2012b). Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. *Nat Cell Biol* 14, 1099-1104.
- van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., *et al.* (2005). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* 435, 959-963.
- Van Raay, T.J., Coffey, R.J., and Solnica-Krezel, L. (2007). Zebrafish Naked1 and Naked2 antagonize both canonical and non-canonical Wnt signaling. *Developmental biology* 309, 151-168.
- van Veelen, W., Le, N.H., Helvensteijn, W., Blonden, L., Theeuwes, M., Bakker, E.R., Franken, P.F., van Gurp, L., Meijlink, F., van der Valk, M.A., *et al.* (2011). beta-catenin tyrosine 654 phosphorylation increases Wnt signalling and intestinal tumorigenesis. *Gut* 60, 1204-1212.

- Vin, H., Ching, G., Ojeda, S.S., Adelmann, C.H., Chitsazzadeh, V., Dwyer, D.W., Ma, H., Ehrenreiter, K., Baccarini, M., Ruggieri, R., *et al.* (2014a). Sorafenib suppresses JNK-dependent apoptosis through inhibition of ZAK. *Mol Cancer Ther* 13, 221-229.
- Vin, H., Ojeda, S.S., Ching, G., Leung, M.L., Chitsazzadeh, V., Dwyer, D.W., Adelmann, C.H., Restrepo, M., Richards, K.N., Stewart, L.R., *et al.* (2014b). BRAF inhibitors suppress apoptosis through off-target inhibition of JNK signaling. *eLife* 2:e00969.
- Voorham, Q.J., Janssen, J., Tijssen, M., Snellenberg, S., Mongera, S., van Grieken, N.C., Grabsch, H., Kliment, M., Rembacken, B.J., Mulder, C.J., *et al.* (2013). Promoter methylation of Wnt-antagonists in polypoid and nonpolypoid colorectal adenomas. *BMC cancer* 13.
- Waalder, J., Machon, O., Tumova, L., Dinh, H., Korinek, V., Wilson, S.R., Paulsen, J.E., Pedersen, N.M., Eide, T.J., Machonova, O., *et al.* (2012). A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. *Cancer research* 72, 2822-2832.
- Waalder, J., Machon, O., von Kries, J.P., Wilson, S.R., Lundenes, E., Wedlich, D., Gradl, D., Paulsen, J.E., Machonova, O., Dembinski, J.L., *et al.* (2011). Novel synthetic antagonists of canonical Wnt signaling inhibit colorectal cancer cell growth. *Cancer research* 71, 197-205.
- Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153, 910-918.
- Wang, R., Wei, Z., Jin, H., Wu, H., Yu, C., Wen, W., Chan, L.N., Wen, Z., and Zhang, M. (2009). Autoinhibition of UNC5b revealed by the cytoplasmic domain structure of the receptor. *Molecular cell* 33, 692-703.
- Wang, Y. (2009). Wnt/Planar cell polarity signaling: a new paradigm for cancer therapy. *Mol Cancer Ther* 8, 2103-2109.
- Wawrzaka, D., Métiouia, M., Willemsa, E., Hendrickxa, M., Genstb, E.d., and Leynsa, L. (2007). Wnt3a binds to several sFRPs in the nanomolar range. *Biochemical and Biophysical Research Communications* 357, 1119-1123.
- Weise, A., Bruser, K., Elfert, S., Wallmen, B., Wittel, Y., Wohrle, S., and Hecht, A. (2010). Alternative splicing of Tcf7l2 transcripts generates protein variants with differential promoter-binding and transcriptional activation properties at Wnt/beta-catenin targets. *Nucleic Acids Research* 38, 1964-1981.
- Westphalen, C.B., Asfaha, S., Hayakawa, Y., Takemoto, Y., Lukin, D.J., Nuber, A.H., Brandtner, A., Setlik, W., Remotti, H., Muley, A., *et al.* (2014). Long-lived intestinal tuft cells serve as colon cancer-initiating cells. *J Clin Invest* 124, 1283-1295.
- Wetering, M.v.d., and Clevers, H. (1992). Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. *The EMBO journal* 11, 3039-3044.

- Wetering, M.v.d., Verweij, C., Hurlstone, A., Batlle, E., Haramis, A.-P., Moerer, P., Soete, G., Medema, R., Sancho, E., Lau, W.d., *et al.* (2002). The b-Catenin/TCF-4 Complex Imposes a Crypt Progenitor Phenotype on Colorectal Cancer Cells. *Cell* 111, 241-250.
- WHO (2014). World Cancer Report 2014. In World cancer report, W.H. Organization, ed. (Lyon: World Health Organization).
- Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448-452.
- Wong, H.-C., Bourdelas, A., Krauss, A., Lee, H.-J., Shao, Y., Wu, D., Mlodzik, M., Shi, D.-L., and Zheng, J. (2003). Direct Binding of the PDZ Domain of Dishevelled to a Conserved Internal Sequence in the C-Terminal Region of Frizzled. *Molecular cell* 12, 1251–1260.
- Wong, J., Smith, L.B., Magun, E.A., Engstrom, T., Kelley-Howard, K., Jandhyala, D.M., Thorpe, C.M., Magun, B.E., and Wood, L.J. (2013). Small molecule kinase inhibitors block the ZAK-dependent inflammatory effects of doxorubicin. *Cancer biology & therapy* 14, 56-63.
- Wong, V.W.Y., Stange, D.E., Page, M.E., Buczacki, S., Wabik, A., Itami, S., Wetering, M.v.d., Poulson, R., Wright, N.A., Trotter, M.W.B., *et al.* (2012). Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nature Cell Biology* 14, 401-408.
- Wu, J., Roman, A.C., Carvajal-Gonzalez, J.M., and Mlodzik, M. (2013). Wg and Wnt4 provide long-range directional input to planar cell polarity orientation in *Drosophila*. *Nat Cell Biol* 15, 1045-1055.
- Xu, Q., Wang, Y., Dabdoub, A., Smallwood, P.M., Williams, J., Woods, C., Kelley, M.W., Jiang, L., Tasman, W., Zhang, K., *et al.* (2004). Vascular Development in the Retina and Inner Ear: Control by Norrin and Frizzled-4, a High-Affinity Ligand-Receptor Pair. *Cell* 116, 883-895.
- Yamada, M., Udagawa, J., Matsumoto, A., Hashimoto, R., Hatta, T., Nishita, M., Minami, Y., and Otani, H. (2010). Ror2 is required for midgut elongation during mouse development. *Dev Dyn* 239, 941-953.
- Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S., and Kikuchi, A. (1999). Phosphorylation of Axin, a Wnt Signal Negative Regulator, by Glycogen Synthase Kinase-3 β Regulates Its Stability. *The Journal of biological chemistry* 274, 10681–10684.
- Yamamoto, H., Kishida, S., Uochi, T., Ikeda, S., Koyama, S., Asashima, M., and Kikuchi, A. (1998). Axil, a Member of the Axin Family, Interacts with Both Glycogen Synthase Kinase 3 β and β -Catenin and Inhibits Axis Formation of *Xenopus* Embryo. *Molecular Cell Biology* 18, 2867-2875.
- Yamamoto, H., Yoo, S.K., Nishita, M., Kikuchi, A., and Minami, Y. (2007). Wnt5a modulates glycogen synthase kinase 3 to induce phosphorylation of receptor tyrosine kinase Ror2. *Genes Cells* 12, 1215-1223.

- Yan, H.H.N., Lai, J.C.W., Ho, S.L., Leung, W.K., Law, W.L., Lee, J.F.Y., Chan, A.K.W., Tsui, W.Y., Chan, A.S.Y., Lee, B.C.H., *et al.* (2017). RNF43 germline and somatic mutation in serrated neoplasia pathway and its association with BRAF mutation. *Gut* 66, 1645-1656.
- Yan, K.S., Chia, L.A., Li, X., Ootani, A., Su, J., Lee, J.Y., Nan Su, d.Y.L., Heilshorn, S.C., Amieva, M.R., Sangiorgi, E., *et al.* (2012). The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Proceedings of the National Academy of Sciences of the United States of America* 109, 466–471.
- Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., I., L., Woodgett, J.R., and Templeton, D.J. (1994). Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* 372, 798-800.
- Yang, H., Wang, H., and Jaenisch, R. (2014). Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. *Nature protocols* 9, 1956-1968.
- Yang, J.J. (2002). Mixed lineage kinase ZAK utilizing MKK7 and not MKK4 to activate the c-jun N-terminal kinase and playing a role in the cell arrest. *Biochemical and Biophysical Research Communications* 297, 105-110.
- Yang, J.J., Lee, Y.J., Hung, H.H., Tseng, W.P., Tu, C.C., Lee, H., and Wu, W.J. (2010). ZAK inhibits human lung cancer cell growth via ERK and JNK activation in an AP-1-dependent manner. *Cancer science* 101, 1374-1381.
- Yang, W., Xia, Y., Ji, H., Zheng, Y., Liang, J., Huang, W., Gao, X., Aldape, K., and Lu, Z. (2011). Nuclear PKM2 regulates b-catenin transactivation upon EGFR activation. *Nature* 480, 118-122.
- Yilmaz, O.H., Katajisto, P., Lamming, D.W., Gultekin, Y., Bauer-Rowe, K.E., Sengupta, S., Birsoy, K., Dursun, A., Yilmaz, V.O., Selig, M., *et al.* (2012). mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. *Nature* 486, 490-495.
- Yu, H., Seah, A., Herman, M.A., Ferguson, E.L., Horvitz, H.R., and Sternberg, P.W. (2009). Wnt and EGF pathways act together to induce *C. elegans* male hook development. *Developmental biology* 327, 419-432.
- Zallen, J.A. (2007). Planar polarity and tissue morphogenesis. *Cell* 129, 1051-1063.
- Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., and He, X. (2005). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* 438, 873-877.
- Zeuner, A., Todaro, M., Stassi, G., and De Maria, R. (2014). Colorectal Cancer Stem Cells: From the Crypt to the Clinic. *Cell stem cell* 15, 692-705.
- Zhang, X., Yao, X., Qin, C., Luo, P., and Zhang, J. (2016). Investigation of the molecular mechanisms underlying metastasis in prostate cancer by gene expression profiling. *Exp Ther Med* 12, 925-932.
- Zhang, Y., Liu, S., Mickanin, C., Feng, Y., Charlat, O., Michaud, G.A., Schirle, M., Shi, X., Hild, M., Bauer, A., *et al.* (2011). RNF146 is a poly(ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. *Nat Cell Biol* 13, 623-629.

Zhu, L., Gibson, P., Currie, D.S., Tong, Y., Richardson, R.J., Bayazitov, I.T., Poppleton, H., Zakharenko, S., Ellison, D.W., and Gilbertson, R.J. (2008). Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* 457, 603-607.